

**Human Leucocyte Antigen G Expression in Cytomegalovirus
Infection in Normal Individuals and Renal Transplant Patients**

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Declaration

All the work presented in this thesis is my own and any other work is appropriately referenced.

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Abstract

HLA-G is a non-classical human MHC I molecule which is abundantly expressed in trophoblast during pregnancy. This molecule is also expressed by several leucocyte subsets but on a low percentage of cells. It can also be secreted in soluble form. The protein is upregulated in several conditions like viral infections, autoimmune disorders and tumours. Several reports have indicated the expression of HLA-G is associated with allograft acceptance and survival. This study was aimed at investigating the expression of HLA-G and its ligand, KIR2DL4, in association with other activation markers, particularly CD103, BAFF and BAFF-R, in relation to CMV antigen stimulation in healthy subjects. Also, HLA-G and KIR2DL4 expression and their association with genotype were studied in renal transplant patients before and after transplantation.

HLA-G was expressed by a significantly higher proportion of CD19+ B cells and CD56+ T cells following stimulation of peripheral blood mononuclear cells (PBMCs) from CMV+ healthy subjects with CMV antigen. Also, sHLA-G levels were significantly elevated in PBMC supernatants from CMV+ subjects stimulated with CMV antigen.

The HLA-G ligand KIR2DL4 was significantly upregulated in IL-2 culture on CD56+ T cells and significantly downregulated in CD56^{bright} cells. Induction of PBMCs with CMV antigens significantly increased the proportion of CD8+ T cells, NK cells and CD56+ cells T cells expressing this ligand. Also, proportions of HLA-G+CD103+ T cells were significantly increased following CMV stimulation.

Stimulation of PBMCs with CMV antigen demonstrated significantly increased proportions of BAFF-R+ CD56+ cells and significantly decreased proportions of BAFF-R+ B cells. HLA-G was expressed on a significantly greater proportion of BAFF+ and BAFF-R+ cells following CMV stimulation.

In renal transplant patients, HLA-G was expressed on a significantly higher proportion of CD4+ cells and monocytes as well as B cells following transplantation. However, KIR2DL4 was significantly upregulated only on CD56^{dim} cells following transplantation.

Finally, culture of PBMCs with the standard immunosuppressive drugs used in transplant patients did not generally inhibit or augment the effects of CMV on HLA-G, CD103, BAFF and BAFF-R expression by PBMCs from healthy subjects, apart from CD4+ cells and CD56+ cells of which significantly greater proportions expressed HLA-G in response to tacrolimus (Prograf) in combination with CMV particles. Also, CD103 was expressed by significantly higher proportions of these cells in the same treatment combination.

In conclusion, the results are consistent with CMV enhancing HLA-G expression on several cell types which may play an important role to allow immune escape during virus infection. In renal transplant patients, proportions of HLA-G+ cells increased significantly in several cell subpopulations after transplantation. Immunosuppressive drug treatment may have contributed to this.

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List of Abbreviation

Ab Antibody

APC Antigen presenting cell

APC Allophycocyanin

ANOVA Analysis of variance

B2m β 2 microglobulin

bp Base pair

BAFF B-cell Activating Factor

BAFF-R B-cell Activating Factor Receptor

CD Cluster of differentiation

cDNA Complementary deoxyribonucleic acid

CMV Cytomegalovirus

CTL Cytotoxic T lymphocyte

CTLA4-ig Cytotoxic T lymphocyte-associated molecule 4-immunoglobulin G1 fusion protein

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DNAse Deoxyribonuclease

DC Dendritic cells

DHODH Dihydroorotate dehydrogenase

DAP12 DNAX Activating Protein of 12KDa

EDTA Ethylenediamine tetra acetic acid

EN Endoplasmic reticulum

ELISA Enzyme linked immunosorbent assay

FasL Fas ligand

FITC Fluorescein isothiocyanate

Fc Fragment crystallisable

FTY720 Fingolimod hydrochloride

FK778 Malononitrilamide 715

FKBP12 FK506-binding protein 12

GB Cytomegalovirus glycoprotein B protein

GH CMV Envelope glycoprotein H

GL CMV Envelope glycoprotein L

HTLV-1 Human T lymphotropic virus type 1

HLA-G Human Leucocyte Antigen-G

HLA-F Human Leucocyte Antigen-F

HLA-E Human Leucocyte Antigen-E

HIV Human immunodeficiency virus

ILT-2 Immunoglobulin like trasnscript-2

ILT-4 Immunoglobulin like transcript-4

IL Interleukin

INF-γ Interferon Gamma

IgM Immunoglobulin M

IgG Immunoglobulin G

ITIM Immune tyrosine-based inhibitory motif

IMPDH Inosine monophosphate dehydrogenase

ISA(TX)247 Calcineurin inhibitor

KIR2DL4 Killer Immunoglobulin Like Receptor 2 Domain Long cytoplasmic tail 4 (KIR2DL4)

mAbs Monoclonal Antibody

MHC Major Histocompatibility Complex

ml Millilitre

mM Millimolar

mRNA Messenger ribonucleic acid

microRNA Micro ribonucleic acid

µg Microgram

NF-κB Nuclear factor-kappa B

µL Microliter

ng Nano gram

NK Natural Killer Cells

Pg Picogram

PBS Phosphate buffer saline

PBMC Peripheral blood mononuclear cells

PCR Polymerase chain reaction

PE Phycoerythrin

PercPCy5.5 Peridinin-chlorophyll protein complex cyanine 5.5

qPCR Quantitative polymerase chain reaction

RNA Ribonucleic acid

sBAFF Soluble B-cell Activating Factor

SLE Systemic lupus erythematosus

sHLA-G Soluble Human Leucocyte Antigen-G

SNPs Single Nucleotide Polymorphisms

TCR T cell receptor

T reg T regulatory cells

TNF- α Tumour necrosis factor-Alpha

TGF- β Transforming Growth Factor-Beta

CHAPTER ONE

INTRODUCTION

1.1. The human immune system and HLA

The human defence mechanism represented by the immune system plays a key role in protection against foreign intrinsic or extrinsic harmful intruders like bacteria and viruses. This function is achieved via specific cells, T cells and B cells, that have acquired the ability to identify these pathogens (Gromme & Neefjes, 2002). The continuous exposure of the body of human beings to harmful microbes and other deleterious molecules can bring the innate and acquired immune response into action. The innate passive immune response can exert its effects at different stages like the access site or within the body, this can be elucidated in the form of a barrier provided by the dermis which can block the access of external microbes to underlying tissues (Spiering, 2015).

1.2. Innate immunity

The innate immune system involves a broad spectrum of actions that mobilise various compartments which belongs to the passive immune system, it encompasses polymorphonuclear cells, macrophages, the complement components and other proteins and cytokines (Ayala García, González Yebra, López Flores, & Guaní Guerra, 2012). The main players of passive immunity that represent the initial barriers to many microbes are polymorphonuclear cells and macrophages which participate in controlling common pathogens (Janeway CA Jr, 2001). Neutrophils which possess phagocytic activity are an essential arm of the innate response that can suppress many infective agents because they are equipped with many lethal mechanisms that could deactivate a wide range of pathogenic intruders (Beutler, 2004). While the macrophage retains the ability of phagocytosis and destroying pathogens, still their role is mainly confined to cytokine and chemokine secretion that can attract other inflammatory cells like neutrophils and monocytes from the circulation to induce inflammation (Beutler, 2004; Janeway CA Jr, 2001). Whereas, the natural killer cells are another player in the innate immune system, it retains cytolytic properties and expresses no receptors dedicated for foreign antigens, however, it is considered as an essential cytokine and interferon secretor for instance, IL-3, GM-CSF, IL-10, TNF- α and INF- γ (Vivier et al., 2011). Also, it plays an important role in degrading malignant (Smyth et al, 2002) and viral infected cells (S. H. Lee, Miyagi, & Biron, 2007). Another key component of the

innate immunity is the complement pathways which embraces soluble and membrane bound proteins, once proper stimuli trigger a specific complement pathway, it drives a series of enzymes that activate the components of the complement which generate C3a and C5a which can provoke pores in the cell surface and chemoattraction leading to deleterious effects in the target microbe (Dunkelberger & Song, 2010).

1.3. Adaptive immunity

Active immunity is triggered via the involvement of immature dendritic cells in the peripheral tissues by engulfing foreign antigen at the site of infection, these cells travel from the marrow and reside in various tissue where they act as surveillance for pathogenic microbes (Janeway CA Jr, 2001). Dendritic cells (DCs) can recognise a wide spectrum of pathogens which can be eliminated by them and some antigenic peptides can be retained in regional lymph nodes to be displayed to T cells. This process activates the DC into a professional antigen presenting cell which allows the stimulation of microbe specific T cells (Janeway CA Jr, 2001). Unlike the innate immune pathway, the main cells are B and T lymphocytes, these express specific receptors, T cell and B cell receptor, that can bind to wide variety of peptides and proteins (Vivier et al., 2011). These lymphocytes represent the acting arms of the adaptive response where T lymphocytes can identify peptides displayed by human leucocyte antigens (Lakkis & Sayegh, 2003) while B lymphocytes can distinguish specific antigenic molecules via their immunoglobulin receptor (Nemazee, 2000). These cells have specific corresponding receptors that allow them to proliferate and differentiate into memory and effector cells that give rise to proper adaptive immune responses. Upon binding of the B cell receptor to pathogen, it transforms to a secretory plasma cell that releases antibodies or immunoglobulin and so the name 'humoral immune response' is derived. Cell mediated immunity is orchestrated via T helper and T cytotoxic lymphocytes, when helper T cells identify peptides derived from foreign sources displayed by the molecule formally known as human leucocyte antigen (HLA) via their T cell antigen receptor (Gromme & Neefjes, 2002; J. Klein & A. Sato, 2000).

1.4. The major histocompatibility complex (MHC)

HLA is also known as the major histocompatibility complex (MHC), allowing screening of the antigens derived from peptides within the cells that can be expressed on the plasma membrane and scanned by T cells (Gromme & Neefjes, 2002; Klein & Sato, 2000). The main function of MHC antigens is concerned with controlling the adaptive immune response and it is well recognised as a target in tissue allograft rejection (Bjorkman et al., 1987). The HLA genes are located on the short arm of chromosome six at position 6p21, and constitute around 36,000 bases (Beck & Trowsdale, 2000). Generally, they comprise three closely positioned regions: HLA class I which involves the genes for classical MHC (HLA-A, -B and -C) that is responsible for coding MHC class I heavy chain (Fig. 1.1), while the MHC class II region contains several HLA A and B gene which are translated into α and β domains (Marsh et al., 2005).

The class II HLA DR region mainly comprises of a DRA gene that code for a constant alpha chain and 9 genes formally known as DRB that are responsible for coding the variable beta domain. Likewise, the DP and DQ regions of HLA, both have single gene that encode for alpha and beta domains in addition to some pseudogenes (S. Y. Choo, 2007). The MHC class III region is not related to antigen presentation and encompasses genes that code for the complement system (factor B, C2 and C4) in addition to the tumour necrosis factor family (Beck & Trowsdale, 2000). The main MHC antigens that are associated with human immunity are MHC class I and II, part of the immunoglobulin supergene family (JAN Klein & Akie Sato, 2000).

HLA molecules act as antigen presenters through their binding to peptides from pathogens and exhibit them on the cell plasma membrane. These peptides are the derivatives of cellular processing of either bacteria or viruses; when these molecules are recognised by specific T cells, the effect could take the form of apoptosis of target cells in the case of viruses, activation of macrophages or antibody production from activated plasma cells to neutralise the intruder microbes (Gallegos, Michelin, Dubner, & Carosella, 2016). Generally, almost all nucleated cells in the human body express MHC class I (Gromme & Neefjes, 2002) which presents intrinsic (self and non-self) antigens to T cytotoxic lymphocyte for surveillance and hence allowing the elimination of abnormal cells (Janeway et al., 2001). While MHC class II expression is limited to macrophages, dendritic cells (DC) and B cells, when these cells display peptides derived from extracellular pathogens like bacteria, the helper T lymphocyte

potentially recognise it and will initiate a cascade of immune responses to eradicate the foreign pathogen (Gallegos et al., 2016; Gromme & Neefjes, 2002). HLA class I is a glycoprotein consisting of a heavy chain that bind to a non-variable beta 2 microglobulin ($\beta 2m$) light chain (Bjorkman & Parham, 1990), the $\beta 2m$ in turn is coded on chromosome 15 (Bjorkman et al., 2005).

The extracellular portion of the heavy domain consists of three segments (alpha 1, alpha 2 and alpha 3) these are attached to the plasma membrane via a transmembrane portion followed by an intracellular tail (Bjorkman et al., 2005; Klein & Sato, 2000). Both HLA class I and II are highly polymorphic and the specificity of class I peptide binding is governed by the diversity provided by allelic variation of amino acids in both alpha 1 and alpha 2 chains. $\beta 2m$ together with the alpha 3 chain form the invariant portion of the complex (Bjorkman et al., 2005). The binding site of the processed peptides in MHC class I comprises of two alpha helices, part of the alpha 1 and alpha 2 domains that form a wall like structure based on a mesh like floor of beta pleated sheet formed by the alpha domains. The alpha helices are then coiled and joined at one end to form a short groove that can host the new processed intrinsic peptide ranging from 8-10 amino acids in length (Bjorkman et al., 1987; Eric W. Hewitt, 2003; J. Klein & A. Sato, 2000). Similarly, the HLA class II family (DQ, DP and DR) are structurally similar and they have a glycoprotein composition that take the heterodimer form of alpha and beta chains that are linked noncovalently, the extracellular domains consist of two alpha 1, alpha 2, beta 1 and beta 2 domains. Those are embedded in the plasma membrane via a transmembrane portion followed by cytoplasmic tails (Engelhard, 1994; J. Klein & A. Sato, 2000).

The binding groove of the MHC class II molecule to some extent shares a similarity with that of class I molecule with a slight difference, the walls are constructed from both alpha 1 and beta 1 domains and the floor of the groove, the beta pleated sheet, is made up from the alpha and beta chains. Also, the two walls of the groove are coiled but the two ends remain open to accommodate larger peptides greater than 12 amino acids in length (Engelhard, 1994; Klein & Sato, 2000). Interestingly, the MHC genes exhibit the highest polymorphism among human genes, these polymorphic sites are mainly limited to the groove that hosts the peptide (J. Klein & A. Sato, 2000). The variability of the sequence of the amino acid in the groove site generates great diversity of the MHC binding pocket that can cope with versatile

peptides (Falk et al, 2006). Each MHC allele has a particular sequence of amino acids that can characterise the binding groove (Falk et al., 2006), also, the anchor residues of the peptide in the groove are largely preserved for each allele (Choo, 2007).

Each HLA class I and II allele binds a specific range of peptide configurations of diverse origin (Engelhard, 1994) for instance, HLA class I is limited to intrinsic peptides which can take the form of self-peptides, as well as virus related peptides, that can be identified by T lymphocytes. However, MHC class II is limited to the binding of proteins that have been derived from extrinsic sources. In the case of MHC class I the displayed peptide on the plasma membrane can be scanned by CD8+ cytotoxic T cells via their T cell receptor (Pamer & Cresswell, 1998). While, recognition of the MHC class II bound peptides is mediated by CD4+ T helper lymphocytes, and the expression of class II molecules is limited to specific immune cells that function as antigen presenters (APC) like monocytes, B lymphocyte, Langerhans cells, as well as DCs. These cells up take extrinsic proteins derived from bacteria or viruses through pinocytosis and form a vesicle that degrade the proteins via the effect of endosomal acid components, the resulted peptides are then coupled with class II complex and transported to be express on the APCs surface (Cresswell, 1994; J. Klein & A. Sato, 2000).

T cell receptors (TCR) on the other hand, have two structural variants in disulphide bond heterodimer that contain alpha-beta (α - β) or gamma-delta (γ - δ) chains (van der Merwe & Davis, 2003). Most of the circulating T lymphocytes (>95%) express an α - β TCR and at the time of TCR interaction with peptide plus MHC molecule, some auxiliary receptors expressed on T cells play a role in improving the binding of the these cells (Choo, 2007). On the contrary, natural killer (NK) cells, a sub set of circulating innate system lymphocytes, function as cytotoxic cells but express no TCR (Parham, 2003). These cells are non-MHC class I restricted but have the capacity to identify and eliminate cells that lack or exhibit low level of MHC class I, especially in the case of some malignantly transformed and virally infected cells (Algarra et al, 2004). However, some cells may be susceptible to NK-mediated destruction in some circumstances when they manage to alert the NK cells via proper signalling. Most NK receptors are ligands for MHC class I which could up or down regulate NK cells through the binding of these receptor ligands to target cells (Parham, 2003).

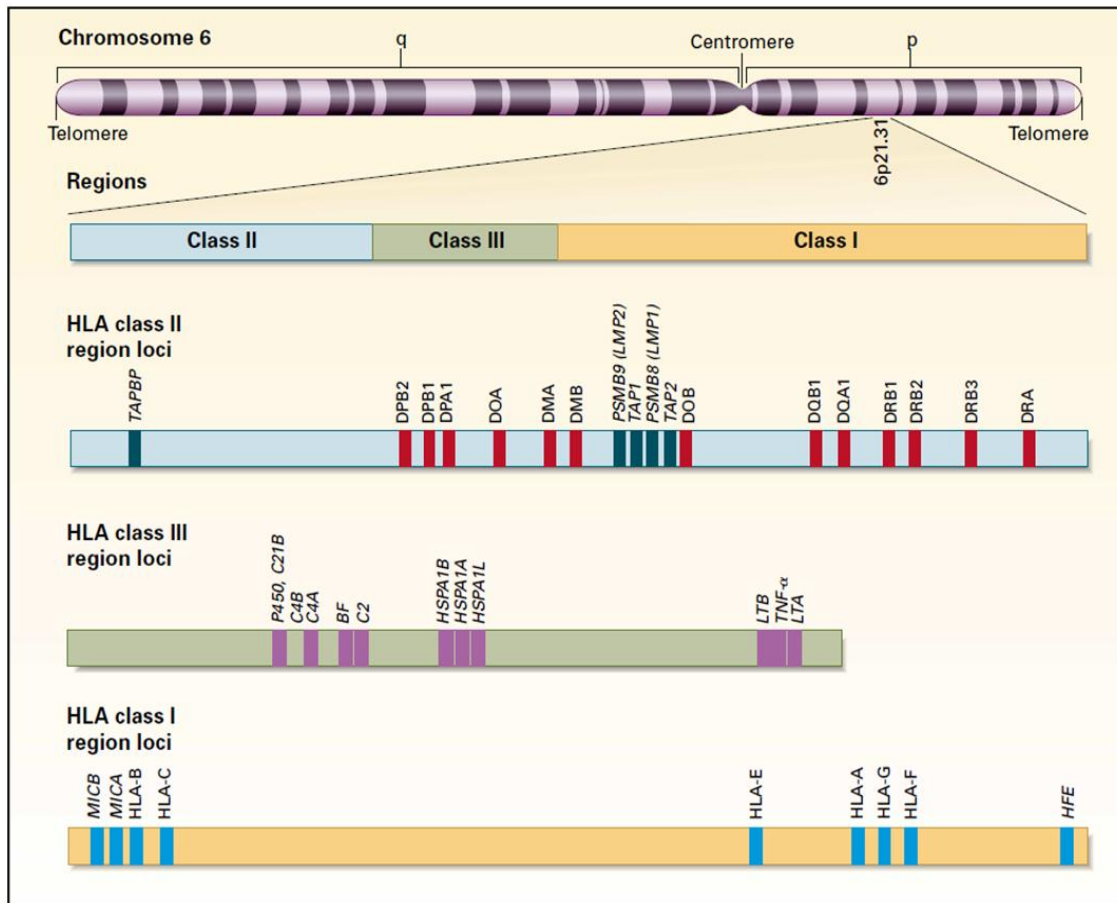


Fig.1.1. HLA loci on human chromosome 6 (J. Klein & A. Sato, 2000).

1.5. MHC class I

The human leucocyte antigen (HLA) class I genes are located on chromosome six (Geraghty et al., 1992) and they share similar basic structure and possess the ability of antigen presentation to other immune cells (Sullivan et al, 2006). MHC class I molecules can be further categorised to subgroups; involving the classical (HLA-A, HLA-B and HLA-C) and (HLA-E, HLA-F and HLA-G) as non-classical variants accordingly (Gallegos et al., 2016). Equally, the non-classical MHC molecules E, F and G can complex in the form of dimers with 2 beta microglobulin with the ability of presenting antigens to other immune cells (Diehl et al., 1996; Shiroishi et al., 2006). Unlike classical HLA variants which display extensive polymorphism, the non-classical molecules exhibit restricted polymorphic diversity with only two isoforms of

HLA-E and F known so far (Shiina et al, 2009; Ulbrecht et al, 1992). The peptides that are exhibited on HLA class I are mostly generated from ribosomal defective products that have been processed by the proteasome in the cytoplasm (Princiotta et al., 2003) which facilitates rapid identification of foreign proteins, e.g. from viruses within the cell to be presented to cytotoxic lymphocyte (Hewitt, 2003). Overall, the presentation process of endogenous peptides by class I MHC molecules is initiated by the generation of cytoplasmic or nuclear peptides by a complex structure named the proteasome. The transporter associated proteins then translocate these peptides from the cytosol to the endoplasmic reticulum where they are incorporated in the peptide binding site of the heterodimer heavy chain complex of MHC class I that is non-covalently bound to beta 2 microglobulin, if they have the correct anchor residues. This process is achieved through chaperones like ERP57, tapasin and calreticulin that form binding sites between the whole complex. The MHC molecule is then exported to the cell membrane via secretory vesicles (Gromme & Neefjes, 2002; Hewitt, 2003).

The classical class I major histocompatibility molecules are encoded by three loci named the HLA region and can display intensive polymorphism with 3,830 HLA-A alleles, while HLA-B has 4,647 alleles and HLA-C has 3,382 alleles (<http://hla.alleles.org/nomenclature/stats.html>) as of December 2016. The HLA locus in humans has the highest gene assembly and a number of these genes participate directly or indirectly in cell mediated immunity by means of encoding for receptors, ligands, mediator proteins, and signalling molecules. Besides, they are involved in the regulation of some fundamental elements of innate immunity such as NK cells and the mobilisation of cytokines (Shiina et al., 2009). Indeed, the HLA molecules play an important role in tissue transplantation, contributing to matching or rejection of tissue or organ transplants (Shiina et al., 2009). The classical MHC molecules A, B and C are groups of highly variable portions that can be displayed virtually by all nucleated cells, the main role of these proteins is to present non self-peptides to T cytotoxic lymphocyte (Zinkernagel & Doherty, 1979). These molecules are implicated in tissue recognition or rejection and this is why the foetus can elude maternal immune surveillance as there is no classical MHC expression on the foetal tissues in early stages of pregnancy (Sunderland, Redman, & Stirrat, 1981). The extravillous cytotrophoblast has no detectable levels of either HLA-A or HLA-B, however, small amounts of HLA-C are expressed on them (King et al., 1996). Added to this, the presence of classical HLA-C and the non-classical HLA-E and HLA-G on

the trophoblast during pregnancy may inhibit the cytotoxic activity of the uterine NK cells when ligation occur with killer Immunoglobulin-like Receptors (KIR) and CD94/NKG2A displayed by these cells (Moffett-King, 2002). On the other hand, in order to elude T cytotoxic recognition, tumour cells tend to inhibit the expression of HLA class I, however these cells might fall under the natural killer surveillance that result in cell death. Thus, the tumour cells might induce other tolerogenic molecules such as non-classical HLA to facilitate the escape from immune inspection (Algarra et al., 2004; Ljunggren & Karre, 1985).

1.6. MHC class II

The major MHC-II group comprises HLA-DR, HLA-DP and HLA-DQ (Gallegos et al., 2016). These fall into a common structure assembled as alpha and beta chains that bind non covalently, which are comprised of alpha 1, 2 and beta 1, 2 domains, transmembrane portion and two alpha and beta cytoplasmic domains (Jones, Fugger, Strominger, & Siebold, 2006; J. Klein & A. Sato, 2000) (Fig. 1.2). Exogenous peptides are drawn into the cell cytosol by infolding of the plasma membrane to form a vesicle that can combine with lysosomes to form phagolysosome that has low pH and proteolytic enzymes that degrade the protein in to smaller subunits for presentation or recycling (Blum, Wearsch, & Cresswell, 2013; Klein & Sato, 2000). In the endoplasmic reticulum, Class II molecules are associated with invariant chain to block endogenous peptide binding which then dissociates on fusion with the phagolysosome. Exogenous peptide replaces the invariant chain in the MHC II complex via the lysosomal support chaperone, HLA-DM, resulting in the formation of antigen MHC-II complexes that are expressed on the plasma membrane for recognition (Castellino et al, 1997; Gromme & Neefjes, 2002). The main role of class II HLA antigen is to display peptides derived from exogenous microbes to helper T lymphocyte which can elicit cellular immune proliferation and activation that lead to the induction of humoral immunity represented by antibody production, B cells antibody class switching and cytoskeletal rearrangement (Jin et al., 2008). Additionally, the expression of MHC-II molecules may signal to promote the process of apoptosis in these cells that have expressed class II complex to T lymphocytes (Jin et al., 2008). In most cases, HLA class II displays peptides derived from extrinsic sources that can be displayed on the surface of specialised cells such as B lymphocyte, monocyte, DCs and macrophages (Castellino et al., 1997;

Gromme & Neefjes, 2002). Also, it can be expressed on non-immune cells like thymic epithelium and in some circumstances, it could be displayed on keratinocytes, epithelial cells and fibroblasts under the influence of cytokines like interferon- γ (Gaspari et al, 1988; Holling et al, 2004). Furthermore, T lymphocytes that have been activated could display a range of HLA class II molecules including HLA-DR, DQ and DP (Holling et al., 2004).

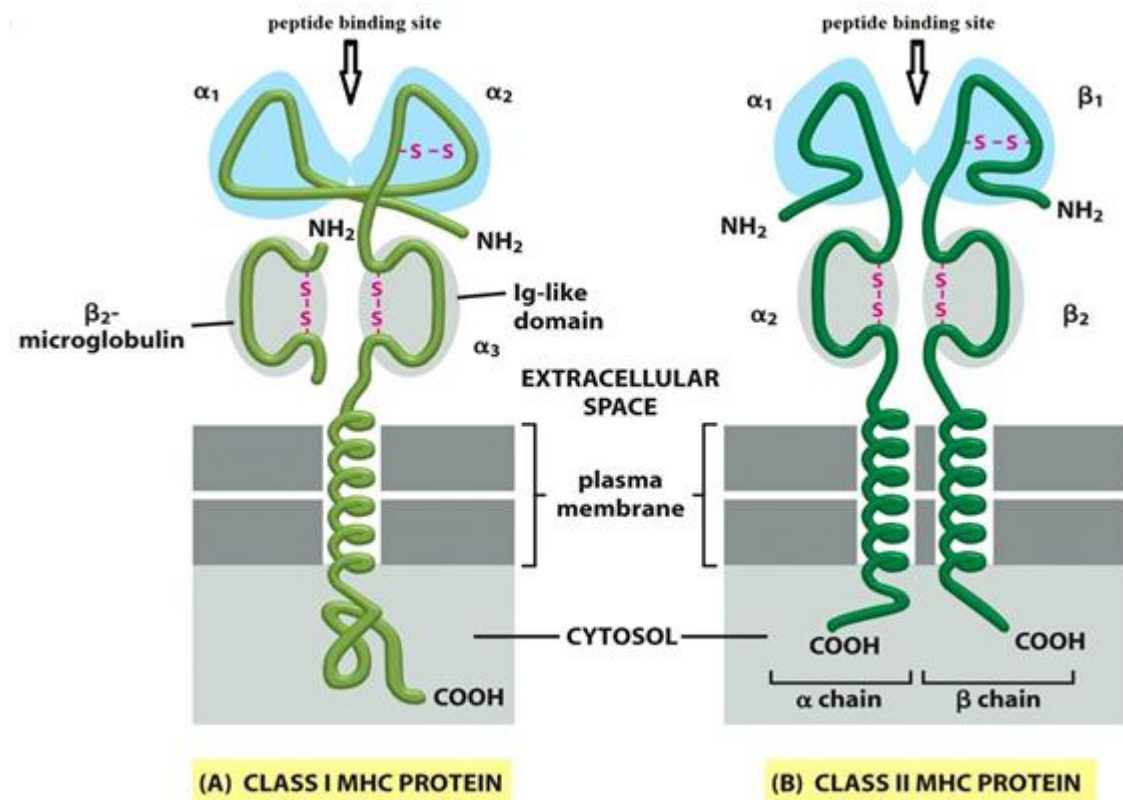


Fig. 1.2. The general structure of the HLA class I and class II molecules (Alberts, 2008).

1.7. Non-classical HLA molecules

The non-classical HLA molecules including MHC-G, MHC-E and MHC-F share the same basic structure with the classical HLA molecules with one major difference is that the polymorphism is very restricted in the non-classical molecules (Ober et al., 1996). In addition, the β_2 microglobulin domain is missing in HLA-F (Goodridge et al, 2010).

1.8. HLA-E

Regarding HLA-E, it shows low heterogeneity with only 13 alleles have been discovered that are translated into 5 different proteins belonging to MHC-E (J. Robinson et al., 2013). Two potential proteins (HLA-E*01:03 and HLA-E*01:01) out of the five have prominent immune effects, these differ in having arginine and glycine at position 107 of the heavy chain alpha 2 domain (Geraghty, Stockschieler, Ishitani, & Hansen, 1992). The roles played by HLA-E implicate the modification of the function of cytotoxic T lymphocytes and NK cells (Pietra, Romagnani, Manzini, Moretta, & Mingari, 2010). Importantly, MHC-E is expressed by various human cells that express class I molecules, binding and presenting a peptide derived from the leader sequence of other class I molecules (Braud et al., 1997); its malformation is associated with some blood disorders (V. Braud, Jones, & McMichael, 1997; Terrazzano et al., 2013). Under normal condition HLA-E is expressed on a variety of human cells including T cells, B lymphocytes, NK cell, megakaryocytes as well as monocytes in addition to endothelial epithelium (Coupel et al., 2007) while its expression in some pathological circumstances has been associated with malignant tumours as well as in some cell lines (Derre et al., 2006). A specific receptor located on the surface of NK cells which is generated by C type lectin (NK group2 and CD94) that act as a ligand for HLA-E molecule (Lanier et al., 1998), this molecule is also expressed on decidual NK cells (Verma, King, & Loke, 1997) which has a heterodimeric structure consisting of non-polymorphic disulphide-linked polypeptide CD94 that bind to the NK group A, B or C molecule (Cantoni et al., 1998). CD94/NKG2 A and B have suppressor effects while the remaining CD94/NKG2 C and D have stimulatory function, those molecules are the ligands for MHC class E antigen (Borrego, Ulbrecht, Weiss, Coligan, & Brooks, 1998; Brooks et al., 1999). The ligation of CD94/NKG2A to HLA-E (V. M. Braud et al., 1998) promotes the suppression of NK, $\alpha\beta$ CD8+ and $\gamma\delta$ T lymphocyte by means of disturbing the actin grid of the immune synapses (Masilamani et al., 2006), whereas the coupling of HLA-E to the stimulatory CD94/NKG2C receptor produces no prominent effect (Garcia et al., 2002).

1.9. HLA-F

As far as HLA-F is concerned, it is a member of the group of non-classical MHC-I molecules that has limited polymorphism (Lima et al., 2016), its locus has 22 alleles that are translated as 4 protein variants (HLA-F*01:01, *01:02, * 01:03 and

*01:04) (Pan et al, 2013). HLA-F consists of a heavy chain only that includes the standard HLA-I domains (alpha 1, 2 and 3) with binding groove in addition to the transmembrane portion and an intracellular tail (Goodridge, Burian, Lee, & Geraghty, 2010). The role of HLA-F is not fully identified and it has been proposed that this molecule follows a similar pattern to HLA-G and HLA-E in terms of immune regulation and tolerance (Lima et al., 2016). These functions of MHC-F are grounded on some evidence such as the ability to influence inhibitory molecules of NK cells and monocytes, the immunoglobulin like toll (ILT) 2 and 4, which can exert regulatory effects on immunity (Lepin et al., 2000). Under physiological conditions, HLA-F is expressed in some cells such as B lymphocytes, splenic cells, tonsils, and thymic cells as well as *in vitro* in the HUT37 T cell line (Lee et al, 2010). Albeit, the expression of this molecule is not confined to normal tissues, but it is also can be detected in some tumours and its expression is associated with poor outcome. In gastric cancer, its expression indicates a metastatic form of the disease (Ishigami et al., 2015) while abundant expression of HLA-F in such malignancies like lung carcinoma, oesophageal cancer and breast carcinoma has been related to poor prognosis (Lin et al., 2011; Zhang et al., 2013). Conversely, it has been found that the expression of this molecule is related to the activity of systemic lupus erythematosus, where the higher the expression of HLA-F, the lower the activity of the disease (Jucaud et al., 2016).

1.10. Human leukocyte antigen G (HLA-G)

HLA-G is a pivotal molecule that belongs to the non-classical MHC-I genes. It displays immune regulatory function that are exerted on other effector or regulator cells in response to certain conditions like cancers, viral infection and possibly some immune disorders (Ben Fredj et al., 2016; Geraghty, Koller, & Orr, 1987). The molecule is well recognised as a tolerance-inducing antigen during early gestation due to the role it takes in preventing the harmful effect of the maternal immunity on the foetus (Kovats et al., 1990). The molecule composition mimics classical MHC-I and HLA-E which share the common domains $\alpha 1$, $\alpha 2$, and $\alpha 3$ coupled with $\beta 2m$ (Menier et al., 2003). Nevertheless, some characteristics, such as limited tissue distribution, distinguish HLA-G from other MHC-I proteins and the cytosolic domain of the molecule is shorter than other proteins in the same class (Gonzalez et al.,

2012) in addition to low polymorphic variation compared to classical MHC-I (E. A. Donadi et al., 2011).

HLA-G is composed of a heterodimer of globular heavy and light domains (β_2m) with a molecular weight of 39 kDa. The heavy chain has three extracellular domains (α 1, 2 and 3) that are anchored to the plasma membrane through transmembrane portion that is followed by a short cytosolic tail (Amiot, Vu, & Samson, 2014). Importantly, HLA-G gene transcription can lead to expression of 7 isoforms that can be induced via alternative splicing. In addition, the heavy chains of the molecule can bind to generate dimers by mean of coupling the cysteines at positions 42 and 147 (Boyson et al., 2002). This feature enables HLA-G to form more powerful associations with other receptors with low detachment rate compared to the non-dimer forms (Shiroishi et al., 2006). One more aspect of HLA-G that discriminate it from other MHC-I molecules is the lack of an endocytic motif in the cytosolic domain (B. Park et al., 2001).

The genes responsible for encoding the HLA-G proteins are situated in between MHC-A and MHC-F loci at chromosome six short arm, position 6p21.2-21.3 (Amiot et al., 2014; Koller et al., 1989). HLA-G has 44 alleles that encode 14 vital proteins including the isotypes (J. Robinson, Waller, Fail, & Marsh, 2006). Genetically it shares a common configuration with MHC-I genes that have 8 exons and 7 introns which are translated into globular proteins of the heavy chain (Amiot et al., 2014; Amiot et al, 2015). Those exons are translated into different portions of the HLA-G molecule, for instance, the signalling peptides, are coded by exon one, the surface portion of the heavy chain (α 1, 2 and 3) are coded by exon 2, exon 3 and 4 correspondingly while the intra membrane region and the cytosolic tail are encoded by exon 5 and 6 respectively. The remaining exons 7 and 8 are not translated in the mRNA because of the stop codon that interrupts the next exon translation in exon 6 (Amiot et al., 2014; E. A. Donadi et al., 2011).

Intriguingly, the promoter genes in HLA-G molecule unlike other MHC-I proteins, have some extra enhancer sequences like A, X1 and S and other components that modulate translation (Gobin & van den Elsen, 2000). In addition, some components of the 3' UTR in the HLA-G locus such as poly A signal as well as the AU rich motif may have some impacts on the mRNA splicing, mobilization and turnover rate (E. A. Donadi et al., 2011). A cardinal hallmark of the HLA-G genes is the ability to generate isotypes of proteins that could be anchored to the plasma membrane

including HLA-G1, 2, 3 and 4 in addition to three proteins that can be excreted in plasma including HLA-G5, 6 and 7 (Fig. 1.3). These proteins are the products of the initial transcript of the mRNA that can be alternatively spliced to produce these isoforms (Carosella et al., 2003). HLA-G1 may take a soluble form that is found in plasma due to the effect of proteolytic enzymes that can cleave it from the plasma membrane (Fujii, Ishitani, & Geraghty, 1994).

Indeed, the complete form of the HLA-G molecule is manifested in HLA-G1 as it is fully coded by its mRNA and has the complete set of exons and introns, while HLA-G2 is encoded by all exons apart from exon 3. Similarly, HLA-G3 has most of the exons in its gene but 3 and 4 are absent, while exon 4 is the only one missing in the case of HLA-G4. The membrane proteins are anchored to the plasma membrane due to the presence of exon 5 and 6 that code the transmembrane portion as well as the cytosolic domain. Correspondingly, HLA-G5 is similar to G1 with the exception of retaining intron 4 and is equivalent in HLA-G6 with intron 4 preserved and exon 3 absent; this give rise to soluble isoforms HLA-G5 and 6 because of the stop sequence that exists in intron 4 which blocks the transcription of the anchoring transmembrane region. Finally, intron 2 is present while exon 3 is missing in HLA-G7 (Amiot et al., 2014, 2015; Fujii et al., 1994). In the same way, soluble HLA-G7 is not a membrane bound because of the stop sequence present in intron 2 (Paul et al., 2000).

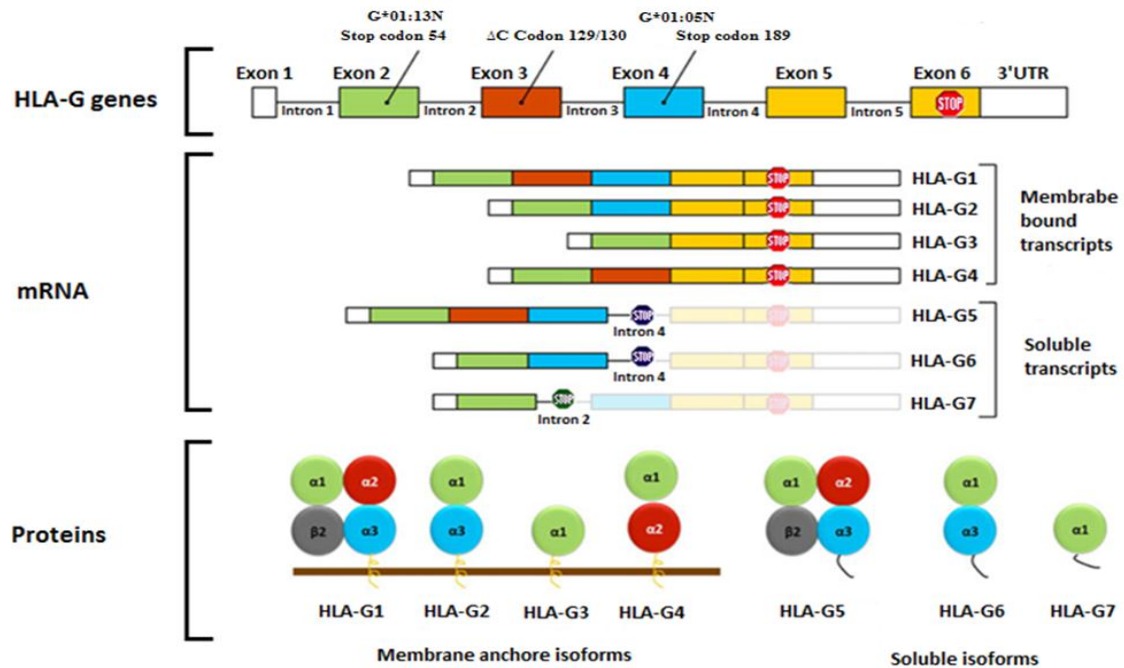


Fig. 1.3. Representative diagram of HLA-G genes that encode for mRNA and the alternative splicing which generate the isoforms of HLA-G proteins (E. A. Donadi et al., 2011).

1.11. HLA-G function

Both the soluble protein and the anchored molecule of HLA-G can induce immune tolerance through interaction with proper regulatory receptors (Amiot et al., 2015). Importantly, the inhibitory molecules that interact with HLA-G proteins are represented by immunoglobulin like transcript ILT2 that is expressed by variety of immune cells such as, NK cells, T cells and B lymphocytes. In addition to ILT4 which is principally expressed on the surface of DC, monocyte and macrophage, the binding of those proteins to HLA-G can modulate the activity of these cells or regulate the proliferative capacity in addition to enhancing the regulation of NK and T lymphocytes (Colonna et al., 1997; Colonna et al., 1998). Similarly, another activator protein, killer immunoglobulin like receptor (KIR2DL4), that act as a ligand for HLA-G may help to tolerate the foetus in pregnancy as it is expressed on decidual NK cells (Moffett-King, 2002). Also, when sHLA-G binds to CD8 protein on the plasma membrane of cytotoxic T cells and NK cells it may lead to induction of apoptosis (Contini et al., 2003; Faure & Long, 2002; Fournel et al., 2000).

The interaction of HLA-G with a variety of immune cells, like granulocytes, T and B cells as well as NK cells, allows this protein to modify or even regulate the innate and adaptive immune responses (Amiot et al., 2014). This is achieved by modifying the process of antigen presentation to T cells, hence delaying the development of DC and impeding their activity (Gros et al., 2008). Added to this, HLA-G can hinder NK and T lymphocyte cytotoxicity along with impairing the differentiation and proliferative activities which leads to inhibition of antibody responses (Le Gal et al., 1999; Naji et al., 2012). On the other hand, the activity of neutrophils can be impaired due to the interaction of HLA-G with ILT4 expressed on these cells which inhibit phagocytic activity and lowers their capacity to release H₂O₂ in response to lung pathogens (Baudhuin et al., 2013).

In the case of malignancies, research has elucidated the role played by HLA-G in eluding immune surveillance (Amiot et al., 2011). In addition, HLA-G expression in plasma and endocardial tissue from individuals with heart transplants has been associated with decreased risk of early graft rejection and subsequent later rejection (Lila et al., 2002). Also, those patients who had liver, kidney or heart transplantation expressed elevated levels of HLA-G had better outcome with low early rejection episodes (Creput, Le Friec, et al., 2003; Lila et al., 2002).

1.12. HLA-G expression

Under normal circumstances, membrane bound HLA-G is expressed on cytotrophoblast, corneal tissue, mesenchymal cells, thymus cells, erythroblasts as well as the matrix of the nails (Amiot et al., 2015; Ben Fredj et al., 2016; Le Discorde, Moreau, Sabatier, Legeais, & Carosella, 2003; Mallet et al., 1999; Torres et al., 2009). On the contrary, the soluble form of HLA-G which is normally synthesised in monocytes occurs in the plasma of healthy individuals (Gros et al., 2006). The molecule has also been reported to be produced by trophoblast at early gestation and detectable quantities have been noted in amniotic fluid (Rebmann et al., 1999; Solier et al., 2002).

In certain diseases, for instance, autoimmune and inflammatory disorders, the expression of HLA-G may help alleviate the condition, while in the case of viral infections or tumours, HLA-G may exert deleterious effect on the patient (Mociornita et al., 2013). Many elements could be influencing the upregulation of HLA-G in vitro, especially cytokines like interleukins 2 and 10, TGF- β , as well as GM-CSF

(Gros et al., 2006). Also, some components may upregulate HLA-G such as, indoleamine 2, 3 dioxygenase, in addition to a decrease in oxygen levels in the microenvironment (Gonzalez-Hernandez et al., 2005; Mouillot et al., 2007).

HLA-G may be induced in viral infection as virus products interrupt cellular expression of some proteins leading to the upregulation of the tolerance mechanism (Amiot et al., 2015). Indeed, herpes simplex I and rabies viruses in humans have the capacity to provoke HLA-G expression in neural cells during active disease (Megret et al., 2007). Also, during the course of infection with influenza or H1N1 epidemic viruses HLA-G was shown to be upregulated on the surface of T cells, monocytes, and regulatory T cells (T reg) (H. X. Chen et al., 2011). Hepatitis B and C viruses can also induce HLA-G on T reg and monocytes (Shi et al., 2011) and in those individuals chronically infected with hepatitis B and C viruses, HLA-G was detected on the surface of biliary tract epithelium and liver cells (de Oliveira Crispim et al., 2012; Souto et al., 2011).

On the other hand, HLA-G has been associated with certain tumours such as liver carcinoma, because of the effect of some factors that can render the active immune system vulnerable allowing tumour proliferation. An example on this is the ligation of HLA-G with ILT4 which may suppress the process of antigen presentation to other effector cells, hence eluding immune surveillance (Amiot et al., 2015). Furthermore, this ligation can decelerate the development of DCs which can impair their function and generate early T reg responses (Basturk et al., 2006). Added to this, the binding of HLA-G to ILT4 that is found on the surface of neutrophils can lower their phagocytic ability, thus compromising the immune system which may provide better chances for tumour survival (Baudhuin et al., 2013).

High levels of HLA-G were also recorded in bile duct carcinomas (Hansel et al., 2005) and elevated sHLA-G protein levels were noted in pernicious phases of hepatocellular carcinoma and its level correlates with relapse and with poor prognosis (Cai et al., 2009). Also, elevated levels of sHLA-G were detected in individuals who suffer from breast and ovarian cancer as well as melanoma (Singer et al., 2003; Ugurel et al., 2001). Principally, plasma levels of sHLA-G secretion tend to be more prominent compared to the membrane attached isotypes, especially in those patients with blood borne malignant diseases (Sebti et al., 2003) such as patients who have been diagnosed with non-Hodgkin lymphomas T or B variant who have higher levels of sHLA-G (Sebti et al., 2003).

1.13. Soluble HLA-G (sHLA-G)

sHLA-G is considered as an anti-inflammatory protein that is synthesised by the placenta and contributes to the process of foetal tolerance during pregnancy (Beneventi, Locatelli, De Amici, Martinetti, & Spinillo, 2016). The major role that this protein delivers is the tolerogenic effect that is implicated in successful conception, and in order to accomplish this, several immune cells may fall under its influence such as cytotoxic T cells, NK cells and T reg. Undeniably, women who develop diabetes during conception tend to have elevated levels of sHLA-G compared to healthy women, while those who are predisposed to spontaneous abortion, pre-eclampsia and embryo restricted maturation seems to have lower levels of sHLA-G (Beneventi et al., 2016).

In contrast, the shed isotype HLA-G1 is associated with impaired capillary formation via blocking of FGF-2 induced angiogenesis (Fons et al., 2006). Also, during assisted fertilisation procedure (IVF), the detection of sHLA-G in the embryo media may give rise to high rates of implantation, while certain conditions like frequent abortion and preeclampsia are associated with lowered expression of HLA-G in the mother's peripheral blood (Rizzo, Vercammen, van de Velde, Horn, & Rebmann, 2011). sHLA-G has also been detected in high levels in patients with certain malignant tumours like breast and ovarian carcinoma, melanoma and glioma (Rebmann et al, 2003), while elevated amounts of sHLA-G were shown in HIV-1 infection (Huang et al., 2010). Interestingly, in solid graft recipients increased sHLA-G protein in plasma has been associated with well tolerated transplants with low latent incidence of rejection and improved prognosis (Creput, Le Friec, et al., 2003). Elevated levels of sHLA-G isotypes were noted in heart transplant patients (Gros et al., 2006) and in renal transplantation its secretion is correlated with better outcome (Basturk et al., 2006). Moreover, patients who had renal allograft procedure with elevated sHLA-G protein manifested with reduced IgG anti HLA antibodies as well as low risk of developing latent graft rejection (Qiu et al., 2006).

1.14. HLA-G polymorphism

The HLA-G gene contains around 72 SNPs that are distributed along exon 1 to intron 6, of which 44 are encoding alleles (Donadi et al., 2011). These alleles encode extracellular and transmembrane portions of HLA-G and these polymorphisms are

predominantly observed in domain $\alpha 1$, $\alpha 2$ and $\alpha 3$ (Robinson et al., 2006). In addition, there are a number of polymorphisms that are located in the 3'UTR that can govern the level of HLA-G expression, Although there is limited polymorphism in the HLA-G proteins, the SNPs may alter the functionality of the molecule regarding generation of isoforms, polymerisation and regulation of cellular immune responses (Donadi et al., 2011). The polymorphism in the 3' UTR promoter could be associated with variations in the expression of HLA-G via allelic regulation pre and post translation (Donadi et al., 2011).

Remarkably, the amount of HLA-G exhibited on the cell membrane or in plasma is influenced by an insertion or deletion of 14 base pairs (rs66554220) located at exon 8 (+2960) that can modulate the translation of mRNA and thus the level of HLA-G (Chen et al., 2008). Substantially, the insertion form of 14 bases in exon 8 of HLA-G (5' ATTTGTTTCATGCCT 3') reflects low levels of HLA-G isoform expression in the trophoblast due to reduced mRNA generation (Harrison, Humphrey, Jakobsen, & Cooper, 1993; Hviid, 2006), while, lack of the 14 bp (deletion) form can give rise to elevated levels of HLA-G proteins as the mRNA becomes more stable (Svendsen et al., 2013). The polymorphic patterns in the 14 bp insertion/deletion are influencing mRNA alternative splicing and the formation of the HLA-G isotypes which could alter the HLA-G expression and its impact during gestation (Hviid et al, 2003; Rousseau et al., 2003) and in females with recurrent spontaneous abortion, there is an elevated incidence of the 14bp insertion variant (Yan et al., 2006). Added to this, the polymorphic patterns in the HLA-G proteins could be a predisposing element for HIV, where females with the allelic variant HLA-G*01:01:08 are considered to be more prone to HIV infection in Zimbabwe, while individuals with allelic variant HLA-G*01:05N are more likely to be considerably less predisposed to HIV infection (Lajoie et al., 2006; Matte et al., 2004). Moreover, there has been a correlation between the deletion variant of the 14 bp on exon 8 and the SNP on the same exon at location +3010 with a C nucleotide having low transplacental HIV-1 infection to the embryo (Aikhionbare, Kumaresan, Shamsa, & Bond, 2006; Fabris et al., 2009). Also, The association of the allelic form HLA-G*01:04:01 with the 14 bp deletion exposes the embryo to risk of HCV from an infected mother, unlike the allelic variant HLA-G*01:05N which is associated with insertion of the 14 bp at exon 8 this could lower the vertical transmission risk (Martinetti et al., 2006). This could be attributed to the fact that people with the allelic variant HLA-G*01:01:01 expresses higher levels of sHLA-G compared to people having HLA-G*01:01:03 as well as

HLA-G*01:05N variants, also, people carrying the HLA-G*01:04:01 allele tend to have more sHLA-G expression in serum than HLA-G*01:01:01 allelic variant individuals (Rebmann et al., 2001).

In individuals with sickle cell anaemia, the occurrence of two C encoding alleles at location +3142 seems to provide resistance to infection with HCV (Cordero et al., 2009) and children with the del/del variant of the 14 bp on exon 8 are more prominent to acute episodes of CMV and elevated viral particle excretion in the urine (Zheng et al, 2009a). However, in patients with thalassemia who require bone marrow grafts the occurrence of del/del at the 14 bp site predisposes them to increased acute rejection episodes (La Nasa et al., 2007), unlike patients receiving heart grafts when the same polymorphism is present it can induce increased sHLA-G level with better cyclosporine response (Torres et al., 2009). On the other hand, the 3' UTR has several SNPs that are associated with differences in HLA-G levels (Fig. 1.4), potentially, the occurrence of +3142 C / G (rs1063320) that can couple with 3 miRNAs (148a, 148b and 152) that can generate either stable mRNA and higher HLA-G levels when they bind to the +3142 G allele or reduced protein in the instance of the +3142 C variant (Veit & Chies, 2009).

Interestingly, the insertion variant of 14 bp is in linkage disequilibrium with two SNPs (+3142G and +3187A) which reflect decreased protein expression and mRNA availability (Hviid et al., 2003). Both alleles, C or G expressed at the +3142 site have been linked with the predisposition to develop different types of asthma related to the amount of HLA-G in those patients (Tan et al., 2007). In Canadian subjects the occurrence of an A nucleotide at the +3187 site accords with high incidence of preeclampsia (Yie et al, 2008) and in vitro studies revealed the association of this SNP with lower HLA-G levels as a result of RNA disintegration (Yie et al., 2008). Those SNPs have also been associated with numerous autoimmune diseases and inflammatory conditions (Tan et al., 2007) for instance, myositis, celiac disease, psoriasis and rheumatoid arthritis (Aractingi et al., 2001; K. S. Park et al., 2006; Veit et al., 2008; Wiendl et al., 2000) as well as inflammatory bowel syndrome (Rizzo et al., 2008).

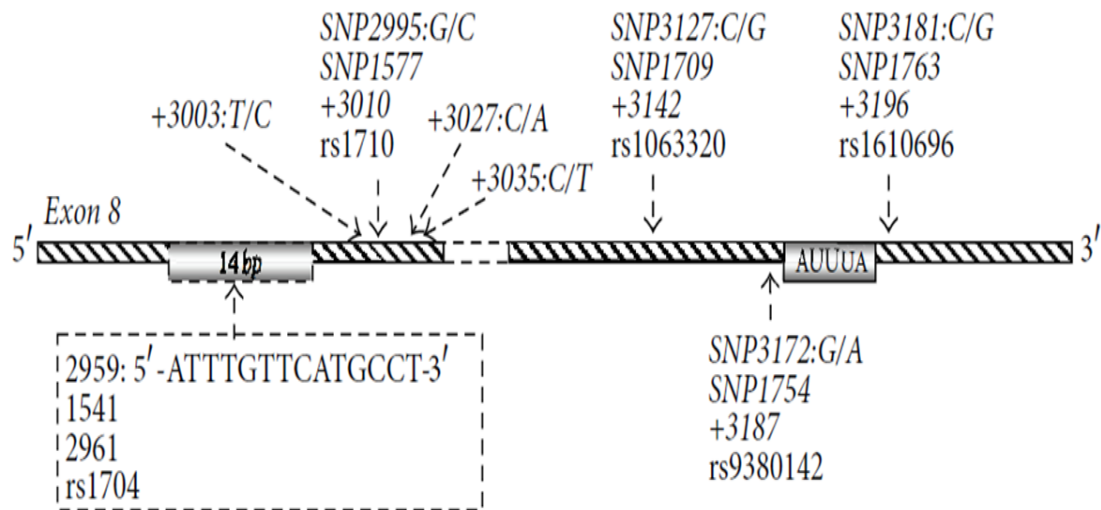


Fig. 1.4. The 14bp and the SNPs location in exon 8 of the HLA-G gene (Dahl, Djuriscic, & Hviid, 2014).

1.15. HLA-G ligand

1.15.1. Killer immunoglobulin-like receptor 2 domain long cytoplasmic tail 4 (KIR2DL4)

NK cells have significant functionality in exterminating transformed and virally infected cells and they can serve as a means of coordination that links the innate and adaptive immune response and they preserve the ability to deliver toxic molecules like granzyme and perforin that can induce cellular lysis. Also, they can exert apoptosis of abnormal cells by exhibiting receptors that provoke autolysis of affected cells like TNF and Fas molecules (Moretta et al, 2000). Moreover, in many inflammatory and infectious diseases they can potentially generate cytokines like TNF- α and INF- γ (Biron et al, 1999) which play a crucial role in the mobilisation of lymphocytes and induce proliferation to deter virally infected or malignant cells (Kikuchi-Maki et al, 2003). Interestingly, killer immunoglobulin-like receptor proteins are a group of receptors that can have both inhibitory and stimulatory function (Vilches & Parham, 2002). These receptors can identify the HLA-I proteins expressed on the cells which in most cases inhibit the stimulation of NK cell (McVicar & Burshtyn, 2001). The family of KIR is categorized according to the

number of domains expressed on the cell surface which take the form of two or three (KIR-2D or 3D) in addition to the cytoplasmic tail which can be either long (L) or short (S), those express inhibitory or activating properties accordingly (Moradi et al., 2015). The receptors that exhibit stimulatory signals have short cytosolic region with lysine in the transmembrane domain with no immunoreceptor tyrosine based activating motif (ITAM), which interacts with DAP12 (Fig. 1.5). The inhibitory receptors have no lysine residue and no DAP12 interaction but they possess a longer cytoplasmic portion as well as two ITIMs (Kikuchi-Maki et al., 2003).

The KIR group is encoded by genes on chromosome 19 (Goodridge et al, 2003) and exhibits extensive variation in alleles and sequence (Hsu et al, 2002). The KIR2DL4 gene is positioned in the centre of the KIR locus and exhibits limited allelic variation (Rajagopalan & Long, 2012). The part of the KIR2DL4 gene which encode the transmembrane region displays a dimorphism in which 9 or 10 adenines are expressed in exon 6 (Rajagopalan & Long, 2012) which in the case of 9A can be translated in the form of a truncated KIR2DL4 protein (J. P. Goodridge et al., 2003) while the 10A form produces mRNA that encodes a full length KIR2DL4 protein that retain the ITIM in the cytosolic domain; the lack of the last adenine in the 9A variant result in minute quantities of mRNA that may encoded for a truncated cytosolic domain with a defective ITIM. This is due to the stop code at exon 7 introduced by the frame shifting when the last adenine is deleted from the ten consecutive As in exon 6 (Goodridge et al., 2003). These allelic variants are equally distributed in the population (Witt et al., 2002).

Importantly, KIR2DL4 has exceptional properties; unlike other KIR groups, the receptor has both stimulatory and inhibitory functions, the transmembrane portion encodes an arginine and the cytosolic portion has only one ITIM (Selvakumar, Steffens, & Dupont, 1996). The KIR2DL4 has two domains displayed on the cell surface including domain 0, and domain 2 but lacks domain 1 (Vilches et al., 2000) and possesses an elongated cytosolic domain (Faure & Long, 2002). The protein has no affinity to bind classical HLA-I molecules and retains the potency to ligate with HLA-G proteins (Ponte et al., 1999) which may result in the production of INF- γ and low cytotoxic effect of NK cells at the fetomaternal interface to allow a proper environment for the developing embryo (Rajagopalan & Long, 1999).

Prominently, KIR2DL4 offers a suppressor receptor site on both uterine and circulating NK cells (Hromadnikova et al, 2013; Ponte et al., 1999; Riteau et al., 2001), in addition, the uppermost transmembrane domain of KIR2DL4 contains

arginine which allows ligation with FcεR1γ that may provoke cytokine release and cytotoxic activity of NK cell upon engagement (Kikuchi-Maki et al, 2005). Despite the suppressing activity exerted by HLA-G on T lymphocytes, yet, the coupling to KIR2DL4 seems to stimulate cytokine production and uterine NK propagation (van der Meer et al., 2007).

At the molecular level, KIR2DL4 mRNA is found in all circulating NK cells (Z. Husain, C. A. Alper, E. J. Yunis, & D. P. Dubey, 2002) and the receptor is exhibited by almost all peripheral NK cells unlike some of the remaining KIR proteins which have sporadic distribution (S. Rajagopalan & E. O. Long, 1999). Conversely, other reports suggested that the KIR2DL4 protein may not be expressed on the plasma membrane of circulating naïve NK cells (J. P. Goodridge et al., 2003; Kikuchi-Maki et al., 2003) because the molecule is located within the endosome (Sumati Rajagopalan & Eric O. Long, 2012). Still, those NK cells with no surface protein may not exert cytotoxic activity but possess robust cytokine production (Rajagopalan, Fu, & Long, 2001) and they retain the signalling from endosomal KIR2DL4 to generate many cytokines like TNF-α, INF-γ, IL-1, 6 as well as IL-8 (S. Rajagopalan & E. O. Long, 2012).

On the other hand, since the proliferation of CMV can take place in endothelial cells and other tissues throughout the body (Ibanez et al, 1991; Sinzger et al, 2008), it's important to elucidate the influence of CMV on lymphocytes migration molecules like Integrin αEβ7 (CD103) which could play an important role in the pathogenesis of the virus and tissue infection.

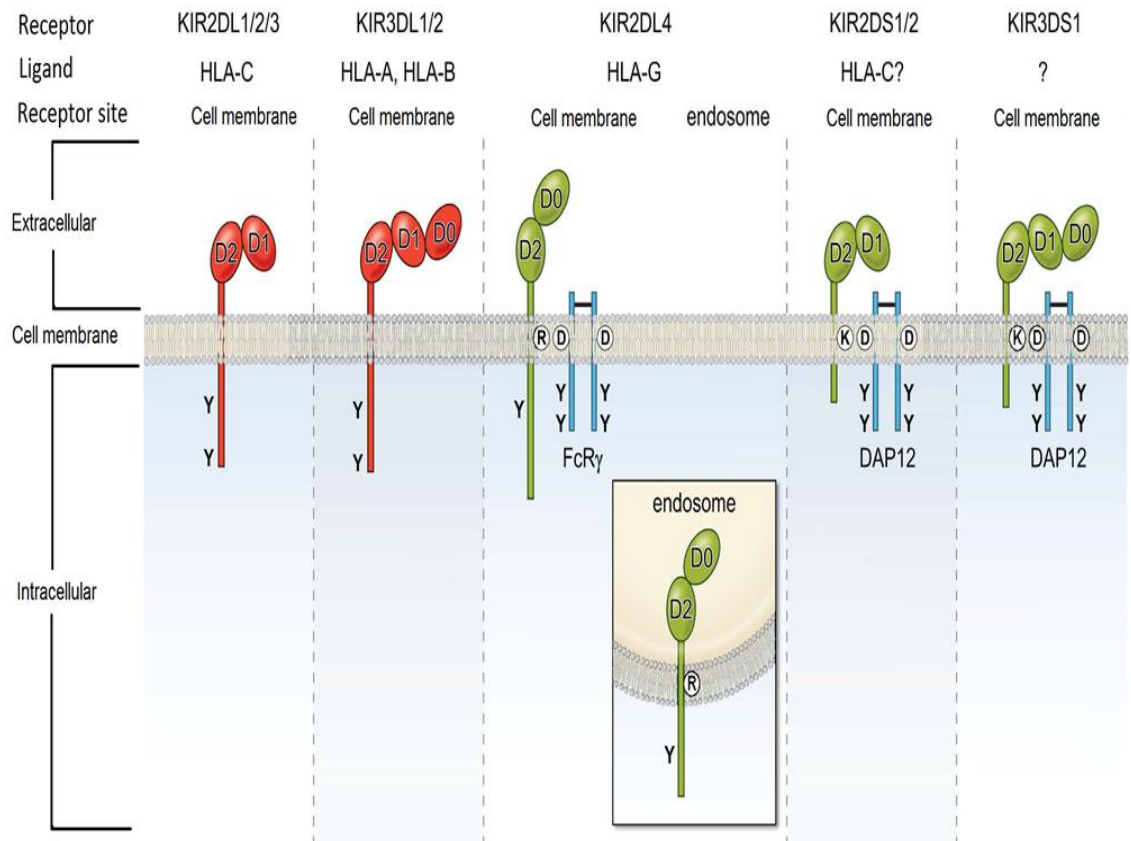


Fig. 1.5. The general structure and distribution of KIR proteins on NK cells and their relevant ligands. The red receptors are activators while the greens are inhibitors (S. Rajagopalan & E. O. Long, 2012).

1.16. Integrin $\alpha E\beta 7$ (CD103)

CD103 is a glycoprotein that belongs to the integrin subfamily, it has a heterodimeric structure and participates in lymphocyte traffic and is confined to mucosal T cells and to lesser extent circulating T cells (Cerf-Bensussan et al., 1987; Hynes, 2002). The function of this protein is to attract lymphocyte to the intraepithelial matrix (Schon et al., 1999), the molecule occurs in roughly 95% of the gut intraepithelial T cells, >40% of the lymphocytes in the lamina propria and nearly 2% of circulating T cells (Parker et al., 1992).

CD103 can be upregulated via TGF- β which can be secreted from epithelial and other cells (Austrup, Rebstock, Kilshaw, & Hamann, 1995) and the molecule has

only one ligand (E-cadherin) that is found on epithelial tissues (Agace et al, 2000). CD103 expression has been associated with CD8 positive T cells that have been found in alveolar fluid, the gut as well as transplanted organs (Rihs et al., 1996; Sarnacki et al, 1992). In addition, this receptor was found to be upregulated in activated T lymphocytes cultured with IL-2 (Brew et al, 1995).

Since the functional compartment of the transplanted organ is the epithelial tissues, the degeneration of such cells by the effects of CD8+ T lymphocytes response can induce rejection episodes (Steinmuller, 1985) as integrin $\alpha\text{E}\beta 7$ facilitate the retention of CD8+CD103+ cells in the graft epithelium which can increase the likelihood of tissue destruction (El-Asady et al., 2005; Feng et al., 2002). This can be elucidated by the presence of effector CD8 T cells infiltrating the donor kidney tubular tissues which is an indicator of early rejection incidence (Racusen et al., 1999) and the presence of E-cadherin, the ligand of CD103, in the epithelial tissue can retain the effector CD8+103+ T cells in the graft tissue and hence more tissue epithelial tissue will be damaged (Karecla et al, 1995; Robertson et al, 2001).

On the other hand, since B cells were shown to express elevated levels of HLA-G (Albayati et al., 2017) following viral induction, it was worth the while to spot the light on the roles played by B cell activating markers in particular the BAFF and BAFF-R during CMV infection.

1.17. B cell activation factor (BAFF)

The role played by B cells in transplantation is associated with antibody induced against the transplanted organ which can provoke early or latent malfunction and subsequent rejection (Stegall et al, 2010). These cells express a cytokine which is a member of the TNF subfamily, BAFF/BlyS, which has the ability to promote the propagation and the life span of B cells as well as antibody release (Mackay et al, 2003; Moore et al., 1999). The molecule is found attached to the cell surface and free in plasma and the main cells that synthesized it are DC, monocytes and macrophages, T lymphocytes in addition to astrocytes and marrow stromal cells as well as stimulated lymphocytes (Daridon et al, 2008; Nardelli et al., 2001).

Three ligands for BAFF have been recognised including BAFF receptor (BAFF-R), B cell maturation antigen (BCMA) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) (Mackay et al., 2003) which are

predominantly displayed on plasma cells and the B cell precursors (Mackay & Leung, 2006). The BAFF molecule generated from the APCs, particularly DC and macrophages has a substantial role in controlling the B lymphocyte reaction toward T lymphocyte independent stimulation (Craxton et al, 2003). The ligand, BAFF-R, which can also be selectively displayed on B lymphocyte (Bossen & Schneider, 2006), acts as a co-stimulatory protein in stimulated T lymphocytes and T reg (Mackay & Leung, 2006; Schneider, 2005).

Elevated plasma BAFF protein has been correlated with many autoimmune rheumatic disorders like Sjögren syndrome and SLE (Groom et al., 2002; Zhang et al., 2001). In kidney transplantation the presence of the BAFF molecule in renal tissues is associated with the precipitation of complement component C4d in recipients with early rejection, moreover, the occurrence of circulating CD4+BAFF+ T lymphocytes seems to be correlated with graft malfunction (H. Xu, He, Sun, et al., 2009). Biopsies extracted from patients with later rejection episodes have been shown to contain high amount of BAFF molecules in addition to increased mRNA expression (Thaunat et al., 2008) and recipients with elevated plasma BAFF titres are substantially prone to develop antibodies to the donor graft (Thibault-Espitia, et al., 2012). Added to this, the level of BAFF in pre transplant recipients is associated with decline of the outcome of the organ and the development of antibody induce rejection (Banham et al., 2013). Likewise, the presence of BAFF-R on the surface of T and B lymphocytes has been correlated with delayed renal rejection episodes (Ye et al., 2004) and in recipients with well-functioning allografts, the increased expression of BAFF-R mRNA in PBMCs has been associated with progression of allograft malfunction (Thibault-Espitia et al., 2012).

1.18. Cytomegalovirus (CMV)

Human CMV is a DNA virus that has a double stranded DNA genome contained in a viral envelope and it is a member of the herpes virus family (CDC, 2010). HCMV belongs to the β -herpesvirinae and is ubiquitously prevalent in human populations at all life stages (Mocarski ES, 2007). Indeed, in the majority of the population, around 50-58%, become infected with the virus by the fourth decade of life (Selinsky et al., 2005) and the worldwide sero-positive CMV distribution varies from 45-100% in adulthood (Gianella, Massanella, Wertheim, & Smith, 2015). Generally, IgG Abs could be detected in around 60% and 100% of populations in industrialised

and unindustrialised countries respectively (Griffiths et al, 2015) and the virus could be detected at early childhood in low socioeconomic areas (P. Griffiths et al., 2015).

The pathogen is capable of inducing persistent infection throughout life, recurrence however is potentially controlled by an effective cell-mediated and humoral immune responses. Nevertheless, immunocompetent individuals may experience disease manifestation in the form of ulceration in the gastrointestinal tract, liver inflammation and in patients with tissue transplantation could develop retinitis and pneumonia (Griffiths et al., 2015).

Particularly, HCMV can remain dormant inside the cells and initiate later infection with sporadic excretion of viral particles (Sinzger et al., 1995), once an infection is acquired in childhood it will remain in the body during the life time due to the effective tactics of the virus to elude surveillance (Effros, 2016). The virus is regarded as a major cause of illness in immunocompromised individuals like HIV and organ allograft patients (Steininger, 2007). In addition, it is considered as one of the significant sources of transplacental infection and the foetus may suffer from congenital abnormalities like visual impairment, hearing problems, fit and microcephalus (Fisher, Genbacev, Maidji, & Pereira, 2000). Gravid females with a primary infection could have 32% risk of embryo infection (Kenneson & Cannon, 2007), and the embryo may develop viremia due to a dormant infected mother and possibly through a different strain infecting the mother (Boppana et al, 2001).

In adults, CMV viremia can cause high grade fever, leukopenia, liver inflammation, gastroenteritis, weakness and pneumonitis (Soderberg-Naucler, 2006). No vaccine has been approved yet for CMV but some commercial medications aimed at impairing the replication of the pathogen are available, though most of them display side effects such as, limited bio-accessibility, increased viral resistance and drug toxicity (Saffert et al, 2010). Ideally, ganciclovir, and cidofovir are frequently used prophylactic medications for CMV in immunocompromised patients and they have shown to be effective and can enhance both life expectancy and wellbeing. Still, these medications have the disadvantages of being cytotoxic and can induce leukopenia (Mocarski et al, 2007).

The structure of CMV represents an ideal herpes virus with the largest viral genome of all human viruses (Mocarski et al, 2007; Stern-Ginossar et al., 2012). its genome consists of 235 Kb of DNA which is contained by a proteinoid capsid that has an

icosahedral shape, the capsid is enclosed by tegument protein and external lipid cover (Mocarski et al, 2007). Individuals infected with HCMV can shed viral particles in various secretions like blood, saliva, urine, seminal fluid, milk from lactating mother and tears, and transmission may be acquired through contaminated saliva, urine, sexual contact, breast feeding and whole blood/blood products transfusion as well as graft transfer and vertical transmission (CDC, 2016).

The virus has the ability to proliferate in a broad spectrum of cells such as endothelium of the vessels, epithelium tissues, hepatic and connective tissues as well as the myeloid derived blood cells particularly macrophages, DCs and fibroblasts (Ibanez et al, 1991; Sinzger et al, 2008). As soon as the virus attaches to the plasma membrane, the access process is initiated which involves the plasma membrane and the glycolipid on the viral envelope, then after, the DNA core plus the tegument are injected into the cytosol (Shenk & Stinski, 2008). Once the virus gains entry, the proteins of the tegument are activated and they will potentially participate in viral amplification, immune evasion, protein expression and viral release (Shenk & Stinski, 2008).

When the virus gains access in to the host cell, the expression of the viral genome occurs at chronological stages as immediate early followed by early gene then late genes. The immediate early genes have an important function in the virion production and later gene expression of the virus particles, these involve UL122 and UL123 (H. Isomura & Stinski, 2003; H. Isomura et al., 2005). While early genes are responsible for encrypting vital proteins required for the reproduction of the virion's DNA (Pari & Anders, 1993), late genes are translated to form the virus structural proteins after DNA reproduction (Hiroki Isomura et al., 2011; Kohler et al., 1994). Some viral genes like immediate early genes within some cells can remain inactive which assume a role in viral persistence and latency (Sinclair & Sissons, 2006). At this stage, the expression of viral proteins and the formation of new virions are kept at a nadir (Shenk & Stinski, 2008).

In certain circumstances, dormant viruses may be reactivated, inducing viremia (Kalejta, 2008). Also, cells that have been infected with CMV retain the ability to generate dense granules and viral envelopes as well as whole pathogenic particles (Kalejta, 2008). The dense granules consist of viral tegument envelope without capsid that has abundant pp 65 proteins, whereas viral envelope resembles a complete CMV virus that has no genomic DNA within (Irmiere & Gibson, 1983).

1.18.1. CMV proteins

Many proteins are coded by CMV in infected cells, some of which are vital for host access like gB, gH and gL glycoproteins which are shared by all members of the herpes family (Connolly, Jackson, Jardetzky, & Longnecker, 2011; Vanarsdall & Johnson, 2012) while other proteins play a role as receptor sites such as UL128, UL130 and UL131 (Haspot et al., 2012).

One of the most ubiquitous viral proteins is pp65 which forms the external part of the virus (Chevillotte et al., 2009). This protein plays an important role in the virus escaping the immune response and also provides a means of immune modification in the course of viral disease (McLaughlin-Taylor et al., 1994). It can modify the adaptive and humoral response and provide epitopes for T cytotoxic cells (McLaughlin-Taylor et al., 1994). This protein is able to hamper the expression of immediate early proteins on MHC-I via the phosphorylation process of those proteins (Gilbert et al., 1996). In addition to its ability to suppress NK cell cytotoxicity (Arnon et al., 2005) through the interaction with the NK p30 receptor on NK cells, preventing elimination of viral infected cells, it also acts to interrupt receptor interaction with DCs and the surrounding NK cells (Arnon et al., 2005).

Another significant protein is (pp71) which participates in initiating the propagation of the virus via activating the expression of immediate early genes to produce new progeny (Spaete & Mocarski, 1985) and its role is to facilitate the viral replication cycle (Kalejta, 2008). In addition, it can prolong the trafficking of MHC-I molecules within the cytosol (Trgovcich et al., 2006), thus delaying the expression of viral peptides on the surface of virally infected cells and evading immune surveillance by T cytotoxic cells (Tomtishen, 2012).

On the other hand, two other viral glycoproteins, pp28 and pp150, possess a pivotal function in the replication cycle, especially in assembly and release of viral particles from infected cells. These proteins can elicit robust immune responses and they have common functionality with some exceptions (Tomtishen III, 2012).

The role of viral protein pp150 is to integrate the synthesised nucleocapsids into the new virions. This protein is copious and ranks second in quantity after pp65 protein (Varnum et al., 2004). Also, this protein functions in trafficking the capsids in the cytosol and preserving their integrity (Tandon & Mocarski, 2008) in addition

to the identification of the assembly unit in the viral formation process (Tandon & Mocarski, 2008).

1.18.2. CMV and immunity

An important aspect of CMV is its ability to influence both the innate and adaptive immune systems in order to minimise the presentation of its peptides to cytotoxic T lymphocytes and elude the immune response (Engel & Angulo, 2012). A specific viral gene, US2-11, has a critical role in blocking the formation of the complex of class I HLA and viral peptides and is also responsible for suppressing the emergence of HLA on the plasma membrane (Benz et al., 2001). In addition to lowering the expression of MHC portions, down regulation of molecules essential for APC mobilization and suppression of T cell activating costimulatory receptors is also mediated by the virus activities (Moutaftsi, Brennan, Spector, & Tabi, 2004). However, the infected cells will be prone to NK lysis when MHC-I is underrepresented on the cell surface, therefore, another strategy of viral evasion works efficiently via the inhibition of NK cell activating ligands and stimulation of the expression of protective molecules like HLA-E on the infected cell (P. Griffiths et al., 2015; Tomasec et al., 2000).

Nonetheless, the primary mechanism that impair viral dissemination in the host is mediated by the cellular immune response (Crough & Khanna, 2009) and the leading players are helper and cytotoxic T lymphocytes, where cytotoxic T cells proliferate and mediate the production of cytokines such as INF- γ to control the infection (Waldman & Knight, 1996). The virus may expose those patients undergoing organ transplantation to the risk of rejection episodes as well as cardiovascular disorders (Rubin, 1989) and the accumulative long standing impact of CMV on immune evasion may exacerbate the mortality rates in those groups (Simanek et al., 2011).

The pathogen could induce haematological tumours in immunocompromised patients with organ allografts, leading to increased morbidity and mortality (Boeckh & Geballe, 2011) this could be attributed to viremia which may lead to end stage organ disease like pneumonia and retinopathy in patients with hematopoietic cell grafts and HIV+ individuals (Evans et al., 2000). In CMV negative patients receiving CMV positive organ grafts, the likelihood of CMV transmission was estimated to be

around 78% (Atabani et al., 2012). Certainly, CMV positive recipients may still be at risk of developing viremia due to the administration of immunosuppressive medication, while CMV positive recipients may also be exposed to different CMV strains from CMV positive donors (Grundy et al., 1988). Also, HCMV can provoke the proliferation of opportunistic retroviruses in patients receiving renal allografts (Bergallo et al., 2015).

As far as CMV control is concerned, viral DNA can be quantified and tracked by mean of polymerase chain reaction (PCR) to determine the viral load in blood in recipients developing viremia post transplantation. The threshold of 3000 particles/ml or (2520 IU/ml) of blood indicates the administration of antiviral medication (P. Griffiths et al., 2015; P. D. Griffiths et al., 2016).

Anti-viral drugs provide efficient mean of prophylaxis in transplant recipients to avoid complications like pneumonia, gut and liver inflammation and retinopathy resulting from viral spread that may accelerate organ rejection (P. Griffiths et al., 2015; Owers, Webster, Strippoli, Kable, & Hodson, 2013; Vanarsdall & Johnson, 2012). Additionally, the normal antiviral regimen involves the administration of ganciclovir for 25 or 50 weeks after transplantation, however some recipients may have viral reactivation after ceasing the medication, particularly in D+/R- patients (P. Griffiths et al., 2015; Owers et al., 2013).

Incidentally, HCMV might be a causative agent for gradual premature decline of the immune system (immunosenescence) which could be manifested by a decline in naïve T helper/cytotoxic cells and increased secretion of IL-6 in plasma which was detected in young carriers of the pathogen (Turner et al., 2014). Moreover, it has been shown that CMV carriers may have elevated counts of CD28- T lymphocytes (Looney et al., 1999). In addition, CMV positive healthy people have a lower ratio of naïve CD8 lymphocytes and an elevated count of CD8 memory lymphocytes regardless of their age (Chidrawar et al., 2009).

The impact of CMV infection is more prominent in patients with immunosuppressant including those undergoing renal transplantation (Steininger, 2007) because such individuals need to be on long protocol of immunosuppressant drugs that could help preventing tissue rejection but in the same time exposing them to increased risk of developing CMV viremia at any point after renal transplantation procedure (Grundy et al., 1988). So, it is an essential aspect to assess the expression of HLA-G in those

patient's and its association renal graft rejection considering the graft obtained from CMV+ or CMV – donors with regards to CMV sero status of transplant recipients.

1.19. Renal transplantation

1.19.1. HLA matching

The active immunity in the graft recipient is a key player in accepting or rejection the graft regardless of the application of immunosuppressive medications (Worthington, Martin, Al-Husseini, Dyer, & Johnson, 2003). Several antigens that are expressed on various cellular components such as the HLA proteins, blood group, as well as monocyte and endothelial antigens could trigger allograft rejection (Ayala García et al., 2012).

The ABO matching is not of such major significance as HLA in the transplant process, however ABO mismatching could progress to hyper acute reactions to heart and renal allografts (Mickelson et al, 1976). Importantly, class I and II MHC molecules have significant effects on the outcome of allograft transplantation (Anasetti et al., 1989) in particularly, the MHC-A, -B and MHC-DR are the basic matching proteins for both recipient and donor and probably HLA-C and HLA-DQ may be included in the matching criteria (Geneugelijk et al, 2017). Following transplantation, the immune response initiated against MHC incompatibility represents a major challenge for the transplanted organs (Opelz & Dohler, 2007) and the susceptibility for allograft rejection is strongly correlated with HLA compatibility (Susal & Opelz, 2013).

Many factors play role in the decision for compatibility of organs for transplant including the lack or the minimal mismatch between MHC-A, -B and MHC-DR for both donor and recipient (Williams et al, 2016). Nevertheless, some other aspects might be influencing the transplant process for instance, the age of the patient, recipient condition, cold ischaemic time and the graft status (Opelz & Dohler, 2007). After organ transplantation, the immune response in the recipient may identify the allo-MHC antigens expressed on donor tissue and this could initiation reaction against it, thus the compatibility of the HLA molecules could have substantial impact on organ survival (Ayala Garcia et al, 2012). The MHC antigens have exclusive epitopes that could be presented on specific molecules and others could be expressed on various MHC groups (Duquesnoy, 2014), the incompatibility between

donor/recipient in the non-shared epitopes could provoke immune reaction in the recipient (Geneugelijk et al., 2017).

Importantly, the assessment of anti HLA antibodies can provide preliminary initial signs of alloimmune reaction against the implanted tissue (Wiebe et al., 2015) and there was pronounced elevation of antibody panels noticed in kidney transplant patients who had graft failure with elevated MHC incompatibility (Meier-Kriesche et al., 2009). Also, the presence of anti-MHC antibodies or elevated CD30 marker on T lymphocytes are correlated with graft survival with regard to MHC compatibility (Susal et al., 2009). In addition, the presence of IgG Abs generated in sensitized renal transplant recipients could complicate the outcome of the transplantation and most of those patients could have a panel of IgM, IgG and IgA anti-HLA Abs (El-Awar et al., 2002), so MHC compatibility could have a substantial positive outcome on the graft acceptance in those patients (Heinold et al., 2013; Takemoto, Terasaki, Gjertson, & Cecka, 2000). Moreover, individuals having sibling grafts matched for MHC may not also be matched at the KIR locus which may affect the survival of the graft (H. Wang et al., 2013).

1.19.2. CMV matching

In organ allograft transplantation, CMV is the most frequently encountered pathogen regardless of the administration of prophylactic drugs (Akalin et al., 2003). Following transplantation, the frequency of developing CMV acute infection (20-60%) is widely affected by the anti-viral medication, the immunocompromised medication and the recipient/donor sero status (Becker et al., 2002) and the infection is more prominent in those donor +/recipient - (D+/R-), also patients receiving lymphocyte depleting medications and insufficient prophylactic antiviral treatment (Becker et al., 2002; Siu et al., 2000).

The infection can cause a major impact on the recipient's wellbeing as well as the transplanted organ (Akalin et al., 2003) through the induction of vascular damage that could initiate acute episodes or latent rejection, nephropathy, atherosclerosis and angiopathic occlusion (Brennan, 2001). Patients receiving organ allografts are predisposing to increased risk of developing CMV infection from CMV positive donors (D+/R-), while D-/R+ in addition to D+/R+ patients are moderately affected by CMV development, whereas the least risk of CMV infection are D-/R- patients

(Humar & Snyderman, 2009). Another risk factors for CMV infection involves the administration of anti-lymphocyte therapy in D+/R- patients and whole blood or blood product transfusion from sero positive donors (Azevedo et al., 2015) and in patients developing graft rejection, the administration of mycophenolate medication and increased corticosteroid dosage (Nemati et al., 2007; Pour-Reza-Gholi et al., 2005). Moreover, diabetes is more likely to arise in patients developing CMV infection following kidney transplantation (Murray & Subramaniam, 2004). Overall, the infection could arise within 12 weeks after transplantation (Schroeder et al., 2004) and its impact is essentially attributed to the cytopathic damage generated by viral proliferation in various organs which could be exhibited in the form of liver inflammation, pneumonitis, retinal inflammation and encephalitis (Azevedo et al., 2015) In kidney transplantation, CMV could elicit immune reaction toward the organ, as excessive use of immunocompromising drugs and acute rejection may induce the activation of CMV infection (Abbott et al., 2002).

1.19.3. Antiviral prophylaxis in renal transplant patients

The introduction of antiviral medications to recipients at increased risk of viral infection for a defined interval after organ implantation is defined as prophylaxis (Azevedo et al., 2015). The incidence of CMV infection in organ recipients basically depends on sero compatibility of the D and R, immunocompromised medication and the organ implanted (Azevedo et al., 2015).

The most widely used antiviral therapies in kidney transplant patients are ganciclovir, valganciclovir and valaciclovir (Azevedo et al., 2015; Kotton et al., 2013; Lumbreras et al., 2014). In addition, in the case of resistant strains of CMV, foscarnet could be administered as a second choice of treatment, however, it is not widely applicable due to the nephrotoxicity and other adverse effect of the drug (Ohta et al., 2001). Albeit, in some cases latent reactivation of CMV can emerge after prolonged administration of antiviral therapy (Humar et al., 2010). In general, the implication of prophylactic antiviral regimen is specified in patients with D+/R- combination accompanied with administration of anti-lymphocyte antibody therapy (Azevedo et al., 2015) and the prophylactic therapy may be initiated post transplantation for 12 weeks or it could be extended depending on the patient's condition (Kotton et al., 2013).

The main point of introducing such antiviral therapy is to suppress virion proliferation and to alleviate the adverse effects of viral disease (Azevedo et al., 2015). The preliminary antiviral medication used in kidney transplantation was acyclovir which later on proved to be not potent in inhibiting CMV disease even at high dose (Kletzmayr et al, 1996).

Valganciclovir has better bioavailability (roughly 70% compared to 7% for ganciclovir) and is derived from a valine ester of ganciclovir (Czock et al, 2002), still, despite the better tolerance and efficacy of drug, its use is restricted due to high price and not being widely accessible. Henceforth, the alternative low cost and broadly accessible ganciclovir is a better alternative (Hertz et al., 1998). This medication has been shown to be potent against serious CMV illness (Crumpacker, 1996) and its administration to recipients with renal transplantation has proven to lower the episodes of CMV infection as well as alleviating the infection severity (Brennan, 2001). While, valaciclovir which is an acyclovir prodrug possesses relatively fair bioavailability, its use is limited because of the low efficacy delivered by the drug and it might be used as an alternative option for treatment (Lowance et al., 1999; Reischig et al., 2012).

1.19.4. Immunosuppressive medications

The aims of administering immunosuppressive medication in organ transplantation are to reduce the lymphocyte count, inhibit or hinder the reaction of lymphocyte to allograft tissue and to alter the traffic of the lymphocytes. These medications function primarily by reducing acute or chronic rejection episodes, however they are not perfect as toxicity and other side effects like infection and perhaps tumour development can emerge (Halloran 2004) such as lymphoproliferative disorders following implantation due to immune impairment (Opelz & Dohler, 2004). In kidney transplant recipients, the potential sources of deterioration and death are heart vascular disorders, infection and malignancies are ranking second and third respectively (Tyden et al., 2009) and infections like pneumonia, urinary tract and viral during the early months of implantation occur more frequently (Alangaden et al., 2006).

Basically, the medications that act on inhibiting the immune response can be classified into three main categories; small molecules, protein therapies and

glucocorticoid drugs as it is summarised in Table 1.1 (Halloran 2004). Some of the widely applicable immunosuppressive medications in renal transplant recipients involve cyclosporine, tacrolimus and mycophenolate mofetil. On one hand, cyclosporine is composed of 11 amino acid that has a cyclic structure which is derived from the fungus (*Tolypocladium inflatam*) (Borel et al, 1976; Marcen, 2009). The molecule couples to cyclophilin which in turn impairs the stimulation of T lymphocytes via calcineurin phosphatase inhibition. Some of the side effects of this drug include elevated lipid profile, nerve intoxication, increased blood pressure, diabetes and haemolytic uremic disorder (Halloran 2004).

On the other hand, tacrolimus is a macrolide derivative which is extracted from *Streptomyces tsukubaensis* (Goto et al., 1987). This component can form a complex with FKBP12 which can suppress T lymphocytes by blocking calcineurin phosphatase, the side effects of this medication are similar to cyclosporine but with lower risk of developing them and more patients are predisposed to nerve toxicity and diabetes following organ transplantation (Halloran 2004). Interestingly, tacrolimus and cyclosporine have the virtue of increasing the graft survival with minimal rejection episodes, but the effect of these medications will not last long as they can induce malfunction in renal tissue (Marcen, 2009). Also, cyclosporine has more lipemic effects (hyperlipidaemia) on treated recipients than tacrolimus (Opelz & Dohler, 2009). Unlike cyclosporine, tacrolimus can significantly provoke diabetes (Burroughs et al., 2007) because of the drug's ability to lower the excretion of insulin in the blood in after use in renal implantation (Vincenti et al., 2007).

In contrast, Mycophenolate mofetil is extracted from penicillium mould and is composed of mycophenolic acid (Mitsui & Suzuki, 1969). The molecule has the ability to inhibit both T and B lymphocyte propagation through impeding the formation of purine and suppressing the generation of the nucleotide guanosine monophosphate. Like other immunosuppressants, it can induce leukopenia, increased bowel motions, and anaemia (Halloran 2004). particularly, the integration of the mycophenolate in addition to corticosteroid and tacrolimus or cyclosporine can provide better prevention against early rejection and long standing organ malfunction with low risk of other undesirable side effects like diabetes, increased blood pressure kidney toxicity and high lipid profile (Marcen, 2009). However, with mycophenolate administration there is a growing risk of CMV development post implantation (Munoz et al., 2002).

Table 1.1. Immunosuppressive medications used in Renal transplantation (Halloran 2004).

- **Glucocorticoids**
- **Small-molecule drugs**
 - 1- Immunophilin-binding drugs:**
 - **Calcineurin inhibitors:**
 - A- Cyclophilin-binding drugs:** cyclosporine, ISA(TX)247.
 - B- FKBP12-binding drugs:** tacrolimus, modified release tacrolimus.
 - **Target-of-rapamycin inhibitors:** sirolimus, everolimus.
 - 2- Inhibitors of nucleotide synthesis:**
 - **Purine synthesis (IMPDH) inhibitors:** Mycophenolate mofetil, Enteric-coated mycophenolic acid, Mizoribine.
 - **Pyrimidine synthesis (DHODH) inhibitors:** Leflunomide, FK778.
 - 3- Antimetabolites:** azathioprine.
 - 4- Sphingosine-1-phosphate-receptor antagonists:** FTY720.
- **Protein drugs**
 - 1- Depleting antibodies (against T cells, B cells, or both):**
 - A- Polyclonal antibody:** horse or rabbit antithymocyte globulin.
 - B- Mouse monoclonal anti-CD3 antibody:** muromonab-CD3
 - C- Humanized monoclonal anti-CD52 antibody:** alemtuzumab.
 - D- cell-depleting monoclonal anti-CD20 antibody:** rituximab.
 - 2- Nondepleting antibodies and fusion proteins:**
 - A- Humanized or chimeric monoclonal anti-CD25 antibody:** daclizumab, basiliximab.
 - B- Fusion protein with natural binding properties:** CTLA-4-Ig (LEA29Y)
 - 2- Intravenous immune globulin.**

1.20. Aims of the study

The current study was conducted to primarily evaluate the following aspects:

- The expression of the non-classical HLA molecule (HLA-G) on various sets of mononuclear cells extracted from healthy donors in response to CMV induction and CMV sero status.
- To establishing a link between HLA-G surface expression and the its gene polymorphisms including the SNPs on exon 8.
- sHLA-G in plasma from the same group of participants was measured to assess whether there is a correlation between surface and soluble form of HLA-G in relation to CMV sero status.
- In order to confirm HLA-G protein expression, molecular analysis (gene expression) was then carried out on of HLA-G and other relevant genes in purified B cell populations stimulated with and without CMV proteins.

- The HLA-G ligand, KIR2DL4, was studied after induction with IL-2 culture to assess the cell surface levels on NK cells and other lymphocyte groups isolated from normal individuals as the HLA-G was noted to be induced in stimulated cells including NK cells and other lymphocytes. In addition, the polymorphism of KIR2DL4 genes on exon 6 was taken into account when involving healthy donors in the study.
- The effect of CMV on the expression of CD103 was studied because of the ability of CMV to proliferate in endothelial and epithelial cells, so it was relevant to assess the influence of CMV on migration molecules to epithelial sites.
- B cell activation factors (BAFF) and its receptor (BAFF-R) were assessed on various mononuclear populations separated from healthy subjects in response to CMV stimulation in vitro. Since, CMV induction was found to increase the amount of HLA-G expressed by B cells, thus it was relevant to study such activating molecules in stimulated B cells.
- In renal transplant patients the expression of HLA-G and its ligand (KIR2DL4) on the surface of mononuclear cells extracted from transplant recipients with known (D/R) CMV status at sequential time points (pre, 2, 4 and 6 months' post transplantation) was carried out to study the changes that may occur after transplantation to those proteins. This is because CMV could increase the risk of tissue rejection and complication in graft function after transplant. Moreover, HLA-G gene polymorphism and the SNPs on exon 8 from transplant patients were also analysed to establish a correlation between these variants and CMV status as well as rejection episodes.
- In accordance with the above sHLA-G in plasma from transplant patients before and after transplantation was also evaluation to see the effect of this protein on transplant outcome and rejection episodes.
- Finally, the influence of immunosuppressant agents (Tacrolimus and Mycophenolate) mainly used in renal transplantation protocol were evaluated to study the changes that may induce by these drugs on the HLA-G, CD103, BAFF and BAFF-R expression in healthy subjects which could be a relevant factor that may suppress or induce those proteins.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Human sampling

2.1.1. Healthy donors

The current study was approved by the University of Liverpool Interventional Ethics Committee (RETH000685) for recruitment of 23 healthy adult donors aged between 21 and 60 and sampling was established in early 2014 until mid 2016. Signed informed consent was obtained from each participant prior to venesection.

Blood samples were collected under aseptic conditions using either heparin or EDTA vials; 20-40 ml was obtained from each donor and further processed for density gradient isolation of mononuclear cells, DNA extraction and plasma separation.

2.1.2. Renal transplant patients

In early September 2015, renal transplant patients were recruited for studying the expression of HLA-G and its ligands after obtaining ethical approval from Haydock NRES Committee North West (15/NW/0351 - HLA-G and its ligand KIR2DL4 in renal transplant patients). The patients were consented and roughly 6-10 ml of blood extracted by the Royal Liverpool Hospital staff, the specimens were submitted for PBMC separation, DNA extraction and also part of the plasma was stored at -20° C. Previously, ethical consent was obtained to acquire archived DNA and sera to study the association of HLA-G genotyping and sHLA-G levels in relation to CMV status and rejection episodes post transplantation (14/NE/0094: Newcastle and North Tyneside NREC, HLA-G expression in renal transplant patients in relation to cytomegalovirus infection).

2.2. Cellular work

2.2.1. Peripheral blood mononuclear cells (PBMCs) separation

Fresh blood (20-40ml) was drawn from each donor under aseptic techniques and aliquoted slowly in 50ml sterile Falcon tubes (Fisher Scientific Inc. USA) containing heparin. The same separation protocol was applied for blood samples obtained from renal transplant patients involved later in the study.

A biosafety cabinet II (MAT-2 CLASS II, Thermo Fischer Scientific Inc. USA) was prepared and sprayed with ethanol 70% (Sigma-Aldrich Inc. Poole, UK) beforehand to allow preparation and later processing of PBMCs as well as cell culturing. All steps performed under careful and standard aseptic conditions to avoid any contamination. The blood was then layered carefully over 1/3 volume of Ficoll Paque Plus solution (Sigma-Aldrich Ltd. UK) using either an automated pipette or manually with a Pasteur pipette with minimal disturbance of the Ficoll. The tube was balanced and centrifuged (Sorvall, Thermo Fischer Scientific Inc. USA) for 30min at 400g with acceleration setting 1 and brake 0 at 18° C.

Following this step, the sample was removed from the centrifuge and the buffy coat layer was recovered with a Pasteur pipette (mostly the focus was on the plasma and the monolayer rather than the Ficoll coat) and aliquoted into a new sterile tube, the volume was completed with PBS (Sigma-Aldrich Inc. UK) up to 40ml and spun at 200g then 150g with brake off.

Cells in the pellet were counted and re-suspended with complete RPMI 1640 plus 2mM L-glutamate media (with 10% FBS and 1% Penicillin-Streptomycin; all components from Sigma-Aldrich Inc. UK) to the required volume necessary for both surface labelling and PBMCs culture.

Plasma samples were separated at the time of processing time and kept at -20° C until further assessments.

2.2.2. Phenotyping

1. Aliquot 500µl of PBMCs cell suspension in 5 FACS tubes.
2. Add 4µl of anti-HLA-G Monoclonal Ab and anti-HLA-DR monoclonal Ab to tube 1-3.
3. Dispense 4µl of anti-human CD14 PerCP-Cy5.5 and PE (BioLegend) to tubes 1 and 2 respectively.
4. Pipette 4µl of anti-human CD1a PE (BioLegend) into tubes 1 and 3.
5. Load 4µl of anti-human CD86 PerCP-Cy5.5 (BioLegend) into tubes 2 and 3.
6. Dispense 4µl mouse IgG1 isotype APC into tube 5.
7. Leave tube 5 without staining as negative control.
8. Incubate the tubes at 4° C for 30 min.
9. Wash the tubes with 2 ml PBS and centrifuge at 500g for 10 min.
10. Decant the supernatant and re-suspend with 500µl PBS.

11. Acquire the cells with Accuri C6 flow cytometer. Adjust the scales of the acquisition plot and gate the DC according to their scatter properties.

2.2.3. Cell line

2.2.3.1. Human choriocarcinoma (JEG-3)

Human JEG-3 cells, originally obtained from the European Cell Culture Collection, were grown as a part of the validation requirement of the anti-HLA-G Ab. These cells are fetal choriocarcinomas that readily express considerable amount of HLA-G and they are relatively easy to grow and maintain and were used as positive control for HLA-G expression. The process of cell seeding and maintenance is described below.

2.2.3.1.2. Cell seeding

1. Remove the required cell vials from liquid Nitrogen.
2. Place the vials in a box filled with ice
3. Transfer the cells to a water bath set at 37° C to allow rapid thawing of the pellets. All the steps were performed in biosafety cabinet II (MAT-2 CLASS II, Thermo Fisher Scientific) sprayed with 70% Ethanol.
4. Pipette the contents of the cryo-vials (SarLab Internationals GmbH, Germany) into 50ml Falcon tube containing 30ml RPMI-1640 complete media.
5. Centrifuge for 5 min at 500g.
6. Discard the supernatant and re-suspend the cells with 30ml complete media.
7. Repeat step 5 and 6 once again.
8. Re-suspend the pellets in 20 ml complete media and, mix by pipetting up and down and distribute an equal volume of cell suspension into two T25 tissue culture flasks.
9. Agitate the flasks gently to ensure parallel dispersion of cells on the plate base.
10. Incubate the plates for a few days at 37° C with 5% CO₂.
11. Observe the growth of the pellets every day, when 90% confluency achieved, passage the cells in two new flasks.

2.2.3.1.3. Cell passage

All procedure performed under aseptic condition in a biosafety cabinet II following the provided protocol (Sigma Aldrich).

1. When the cell confluence approached roughly 90%, pipette the maintenance media off the flask.
2. Add 1-2 ml cell dissociation buffer or trypsin (Sigma Aldrich Inc. UK) for no longer than 2-3 min.
3. Tap the sides of the flask while swirling the flask to facilitate cell detachment.
4. Pipette 20ml of complete RPMI media to deactivate the effect of trypsin.
5. Aspirate the suspension and wash with complete RPMI 1640 media for 5 min at 500g.
6. Repeat the washing once again.
7. Re-suspend the pellet with 20 ml media and equally distribute in two new T25 flasks.
8. Incubate the flasks for a few days at 37° C with 5% CO₂.
9. Inspect the plates every day to observe the growth rate.

2.2.3.1.4. Cell freezing

The extra passage flasks were dedicated for cell preservation by freezing in liquid Nitrogen.

1. When the cells reached 90% confluency, decant the media and add 1-2 ml cell dissociation buffer or trypsin for 1-3 min.
2. Agitate the flask to allow cell dissociation.
3. Dispense 20 ml complete RPMI media to deactivate the effect of trypsin.
4. Pipette the suspension into a 50ml sterile Falcon tube.
5. Aliquot 30 ml complete media and centrifuge the pellet at 500g for 5 min.
6. Repeat the washing in step 5.
7. Prepare 10 ml storage media on ice by mixing 60% FBS (Sigma Aldrich Inc. UK) with 10% DMSO (Sigma Aldrich Inc. UK) and 30% complete RPMI media.
8. Count the cells and detect the viability with Trypan Blue (Sigma Aldrich).
9. Re-suspend the pellets with freezing media and vortex, distribute 4×10^6 cells/2ml in each cryo-vial tube.

10. Transfer the tubes to Mr. Frosty pack (Thermo Fischer Scientific Inc. USA) and leave the cells for two days at -70° C.
11. Move the cryo-vial tubes from Mr. Frosty container to liquid Nitrogen in the specified stack.
Keep record of the tubes and the stack in liquid Nitrogen for later recovery.

2.2.3.1.5. JEG-3 labelling

To validate the anti-HLA-G Ab, JEG-3 cells were phenotyped, washed and acquired with the Accuri C6 flow cytometry.

1. Recover some JEG-3 cells from one of the flasks when reaching 90% confluency.
2. Add 1-3 ml cell dissociation buffer for 1-3 min.
3. Agitate the flask thoroughly to detach the cells.
4. Add 30 ml complete RPMI media.
5. Aspirate the cell suspension into 50 ml sterile Falcon tube.
6. Spin the pellet at 500g for 5 min.
7. Repeat the washing step.
8. Re-suspend the cells in 3ml complete RPMI media.
9. Aliquot 500µl of the cells suspension in three FACS tube properly labelled.
10. Dispense 5µl of anti-HLA-G Ab APC (Thermo Fisher Scientific) in tube 1.
11. Dispense 5µl of isotype mouse IgG1 control APC (Thermo Fisher Scientific).
12. Leave tube 3 as control negative without Abs.
13. Incubate the tubes at 4° C for 30 min.
14. After incubation, load the tubes with 2ml PBS and vortex.
15. Centrifuge at 500g for 5min.
16. Decant the supernatant and re-suspend with 500µl PBS.
17. Acquire the cells with the Accuri C6 plus flow cytometer. Made the proper adjustments to gate the cells and determine Ab staining on the x-axis.

2.2.3.2. Human forensic fibroblasts (HFFB)

This cell line was kindly obtained from a colleague at 60% confluency and is considered as one of the cell lines that allow active HCMV proliferation for the purpose of viral amplification and producing Lab stains. The cells were left overnight

at 37° C with 5% CO₂ and the growth rate was monitored. Upon 90% coverage of the T25 flask, the pellets were passaged in two T25 flasks. All procedures were performed in aseptic condition in a class II biosafety cabinet.

2.2.3.2.1. Cell passage

1. Decant the media and add 1-2 ml trypsin for 2-3 min.
2. Agitate by tapping the sides of the culture flask several times.
3. Pipette 20 ml complete DMEM media (Sigma Aldrich Inc. UK) (with 10% FBS, 1% Penicillin/Streptomycin) (Sigma Aldrich) to deactivate the trypsin.
4. Aspirate the suspension into a 50ml sterile Falcon tube.
5. Complete the volume with complete DMEM media to 30 ml.
6. Centrifuge for 5 min at 500g.
7. Repeat the washing again.
8. Discard the supernatant and re-suspend with 30 ml complete DMEM media.
9. Distribute equal volume of the cell suspension in two T75 flasks.
10. Incubate the cells at 37° C with 5% CO₂ for 2-4 day, Inspect the growth each day.

2.2.3.2.2. Cell freezing

At the 90% confluency, the HFFB were prepared to be stored in liquid Nitrogen.

1. Discard the growth media from one flask and dispense 2-3 ml trypsin for 1-3 min.
2. Tap the sides of the flask thoroughly to detach the cells.
3. Pipette 30 ml complete DMEM media to deactivate the trypsin.
4. Aspirate the cell suspension into a 50ml sterile Falcon tube.
5. Spin the cells for 5 min at 500g.
6. Repeat the washing one more time.
7. Count the cells and dilute with freezing media up to 10 ml.
8. Prepare the freezing media, 60% FBS, 6% DMSO and 34% complete DMEM media.
9. Aliquot 2 ml of cell suspension in cryo-vials, labelled and dated.

10. Place the vials in Mr. Frosty container and store at -70° C for two nights.
11. Remove the cells to liquid nitrogen and maintain a record of the stock and details of the storage box.

2.2.4. Human CMV propagation

HFFB were grown for the purpose of HCMV amplification, two viral strains (AD169 and Towne) were kindly provided by the Virology Department at the Royal Liverpool Hospital. The following protocol was applied for HFFB infection, all procedures were performed under aseptic conditions in a class II biosafety cabinet.

1. Seed HFFB in four T75 flasks.
2. When the cells approximately reach 70-80% confluency, inoculate the cells with viral strains
3. Decant the media.
4. Each strain inoculum was prepared by mixing 250µl of viral stock with 10ml DMEM media (5% FBS with 1% Penicillin/Streptomycin).
5. Inoculate each strain in one T75 flask, leave the remaining two flasks as control.
6. Incubate the inoculated flasks at 37° C with 5% CO₂ for 90 min and make sure that the inoculum is dispersed evenly.
7. Agitate the flasks every 10 min to disperse the viral on the monolayer.
8. Aspirate the inoculum and wash the flasks with PBS twice (by pipetting up and down).
9. Decant the washing PBS.
10. Load 15 ml maintenance DMEM media (5% FBS plus 1% Penicillin/Streptomycin) and mix well.
11. Incubate the flasks at 37° C with 5% CO₂ for two weeks.
12. Inspect the cells every day to check for the development of cytopathic effects (CP).
13. Once the CP develops, aspirate the maintenance media and pour in 50 ml sterile Falcon tube, store at -70° C.
14. Replenish the cells with fresh maintenance DMEM media.
15. Repeat step 13 and 14 every three days.

16. When the cells display extreme CP and degeneration, load 20 ml formalin 20% (Sigma Aldrich) to each flask and incubate for 2 hours then discard the flasks properly.
17. The volume generated from media collection was equally dispensed in sterile ultra-centrifugation tubes (Thermo Fisher Scientific) and weighed to allow maximum difference of 0.1g between tubes.
18. Centrifuge the tubes at 13000g (Ultra centrifuge, Thermo Fisher Scientific) for 3 hours at 3° C.
19. Aspirate the supernatant and re-suspend the viral particles in 1-2 ml maintenance DMEM media containing 1% DMSO.
20. Freeze the vials at -70° C for later use.

2.2.5. PBMC culture

Following separation, a 6, 12 or 24 well flat bottom tissue culture plate (Thermo Scientific Inc. USA) was prepared for the resuspended cells subject to the type of experiment and the number of samples required. Each well was aliquoted with (4×10^6 cell/2ml) to cover the number of samples required for phenotyping including the control as well as cell isolation and RNA extraction.

2.2.5.1. PBMC culture with CMV antigen

The plate designed for culture with CMV antigens was set up for CMV protein-stimulated and control non-stimulated wells. Within the stimulated wells, 1µg/ml CMV strain AD169 protein extract (product code: SUN201, BioRad Inc. USA), this portion was utilized in most of the stimulation experiments and it was validated through the use of serial dilution of the viral protein to induce known number of cells for few days, the stimulation was observed via flow cytometry and the lower in the protein concentration in the well, the lower cell stimulation was recorded. A thorough optimization was performed to obtain the best concentration for PBMCs induction. The protein was added and mixed by pipetting up and down, while the remaining wells were left non-stimulated as a control. Some of the wells (control and induced) were dedicated for RNA extraction and cell isolation (the concentration of CMV lysate was optimised using serial dilution of the viral protein versus PBMCs to evaluate the optimal stimulation within a few days of culture). Afterwards, the plate was incubated for 7 days at 37° C at 5% CO₂.

By the end of day 7, the pellets were aspirated from the wells and aliquoted into several FACS tubes to be labelled with monoclonal antibodies to assess the level of HLA-G on the PBMC subsets. The remaining wells were utilised for RNA extraction, cell isolation and the supernatant from stimulated and non-stimulated wells was kept at -20° C until further used with sHLA-G and sBAFF ELISA.

Likewise, the same setup of culture and CMV concentration was applied to evaluate CD103, B cell activation factor (BAFF) and B cell activation factor receptor (BAFF-R) expression at a different time point.

2.2.5.2. IL-2 culture

KIR2DL4, the ligand of HLA-G, was assessed on the surface of NK cell subsets. PBMCs recovered from healthy donors via Ficoll gradient separation, were cultured in 6 well flat bottom plates with or without IL-2 (R&D System Inc. UK). Each well contained (4×10^6 cells/3ml), IL-2 was dispensed into 3 wells at a concentration of 20ng/ml while the remaining unstimulated wells were left as controls. Some of the wells were designated for RNA extraction, each stimulated well was thoroughly pipetted up and down to ensure even distribution of IL-2. The plate was incubated at 37° C for two weeks in the presence of 5% CO₂, during which the media was replenished every 3-4 days with 2ml fresh complete RPMI 1640 media for the negative control and with 20ng/ml IL-2 for the stimulated wells.

2.2.6. Effect of immunosuppressive drugs on HLA-G expression

The impacts of Prograf (Tacrolimus) and Mycophenolate Mofetil, the common immunosuppressive medications that are used with live transplant patients pre and post operation, on the expression of HLA-G and its ligands were evaluated on PBMCs from healthy donors. The standard physiological therapeutic concentration of the drugs was used *in vitro* in the culture media in the following combinations; control non-treated well, CMV particles alone well, Prograf plus CMV particles, Mycophenolate plus CMV particles, Prograf and Mycophenolate plus CMV particles in addition to Prograf and Mycophenolate without CMV particles. Fresh PBMCs were extracted as previously stated (3.3) and the cells were cultured at 10×10^6 cell/5ml. The viral particles extracted (3.14) were optimised by culturing the PBMCs with serial dilutions in a separate experiment to determine the best concentration that provokes cell proliferation. The stock Prograf was diluted to a standard

concentration of 10ng/ml, while the Mycophenolate was used at 3µg/ml. Standard aseptic conditions were used in a class II biosafety cabinet.

Procedure

1. Extract the PBMCs from 40 ml fresh blood.
2. Wash the cells with PBS or complete RPMI media twice.
3. Discard the supernatant and re-suspend the pellets with 10 ml complete media.
4. Count the cells and dilute to appropriate volume with complete media.
5. Aliquot 10×10^6 cell/5 ml in each well of 6 wells plate.
6. Dilute the stock Prograf with PBS to 10ng/ml.
7. Prepare a working solution of Mycophenolate at 3µg/ml.
8. Thaw the CMV particles and mix well.
9. Leave well 1 as a control non-treated well.
10. Add 3µl/ml CMV particles to wells 2, 3, 4 and 5.
11. Dispense 10ng/ml Prograf to wells 3, 5 and 6.
12. Pipette 3µg/ml Mycophenolate to wells 4, 5, and 6.
13. Mix each well by pipetting up and down.
14. Incubate the plate at 37° C with 5% CO₂ for 7 days.
15. At day 7 remove the plate out of the incubator and proceed with the staining protocol.
16. Aspirate the cells from each well in a separate 15 ml Falcon tube, wash the cells with PBS once at 400g for 10 min and discard the supernatant.
17. Fix the pellets with 3 ml cell fixation buffer (eBiosciences Inc. USA) for an hour.
18. Wash the pellets with PBS once at 400g for 10 min.
19. Re-suspend the cells with 5 ml PBS.
20. Aliquot the 400 of cells suspension in several FACS tubes and label the cells with HLA-G, CD103 as well as BAFF and BAFF-R as previously mentioned (5.3.1.1, 5.3.1.4 and 5.3.1.5).
21. Acquire the cells with the Accuri C6 flow cytometry and plot for the right Abs in each tube.

2.2.7. Cell isolation

2.2.7.1. B cell separation

Human B cells were isolated from total PBMCs cultured with CMV protein after one week of stimulation as previously described in 3.4.1. A negative selection cell isolation kit (EasySep™ Human B Cell Enrichment Kit, STEMCELL Technologies Inc. Canada) was utilized to separate B cells which were further processed to confirm mRNA gene expression of HLA-G. The kit was found to be very useful to use and can produce very high target cell purity. The kit is based on Abs that target all cell populations in PBMCs except the B cells and magnetic beads which bind to the Abs in the cell suspension. All reagents were ready to use and prior to performing the procedure, the components were brought to RT following the manufacturer's protocol and under aseptic condition in a class II biosafety cabinet.

Procedure

1. Harvest stimulated and non-stimulated PBMCs from culture plate into two FACS tubes.
2. Wash the pellets with PBS or complete RPMI media twice at 400g for 5 min.
3. Decant the supernatant and count the cells, ensure that cell count is $<5 \times 10^7$ /tube.
4. Transfer the cells into a sterile 5 ml FACS tube.
5. Dilute the cells with 1ml PBS and vortex.
6. Add 50µl Ab cocktail, vortex and incubate the cells at RT for 10 min.
7. Thoroughly vortex the magnetic particles.
8. Pipette 75µl magnetic particles into the cells and vortex well.
9. Leave the tube at RT for 5 min.
10. Dilute the sample with PBS up to 2.5 ml and thoroughly pipette up and down.
11. Insert the tube without lid in the EasySep™magnet (STEMCELL Technologies Inc. Canada) in the vertical position.
12. Leave the tube plus the cell suspension in the magnet for 5 min at RT.
13. In one movement, grab the magnet plus the tube inside and invert to pour down the negatively selected B cells into a new sterile 15 ml Falcon tube.
14. Centrifuge the tubes to pellet the B cells and proceed for RNA extraction.

2.2.7.2. NK cell separation

Similarly, NK cells were separated from total PBMCs cultured with IL-2 after two weeks of induction as described earlier in 3.4.2. The cells were isolated with a negative selection kit dedicated for the purpose (EasySep™ Human NK Cell Enrichment Kit, STEMCELL Technologies Inc. Canada). The kit was found to be very productive and easy to use and the output is highly purified target cell. The selected cells were further submitted to RNA extraction to detect the level of mRNA expression for KIR2DL4 in the induced and non-induced NK cells. The kit allows the capture of all PBMC sets except the NK cells with monoclonal Abs that bind to magnetic particles.

The kit components were ready to use and the reagents brought to RT prior to use. All procedures were applied according to the supplier's instructions under aseptic condition.

Procedure

1. Aspirate the PBMCs from both stimulated and non-stimulated wells into two sterile FACS tubes.
2. Wash the pellets with PBS twice at 400g for 5 min.
3. Discard the supernatant and count the cells, ensure that cell count is $<5 \times 10^7$ /tube.
4. Aliquot the cells into a sterile 5 ml FACS tube.
5. Complete cell suspension volume to 1ml with PBS and vortex.
6. Pipette 50µl enrichment Ab cocktail, vortex and incubate at RT for 10 min.
7. Thoroughly vortex the magnetic particles.
8. Load 100µl magnetic particles to the pellets and vortex well.
9. Incubate at RT for 5 min.
10. Complete the sample volume with PBS to 2.5 ml and thoroughly pipette up and down.
11. Insert the tube without cap in the EasySep™ magnet (STEMCELL Technologies Inc. Canada) in a vertical position.
12. Leave the tube plus the cell suspension in the magnet for 2.5 min at RT.
13. In one movement, grab the magnet plus the tube inside and invert to pour down the negatively selected NK cells into a new sterile 15 ml Falcon tube.

14. Centrifuge the tubes to pellet the NK cells and progress to RNA extraction step.

2.2.8. Antibody labelling

The PBMCs extracted from healthy donors at day 0 and following culture with CMV and IL-2 were phenotyped with a panel of monoclonal antibodies (mAbs) corresponding the antigens expressed by defined groups of cells.

Also, PBMCs from renal transplant samples that were extracted at the collection day were submitted to the HLA-G and its ligands, KIR2DL4 and CD103, surface labelling only as described below.

The Abs were selected on the bases of optimal matching of four colours (FITC, PE, PerCP-Cy5.5 and APC) that can suit the BD Accuri C6 flow cytometer (BD Biosciences, Oxford); the main populations tested were T cells, B cells, monocytes, NK cells and Treg.

The flow cytometry is based on labelling mixed cellular culture or cell line with fluorescent Abs that are tagged with various fluoresceins that could be illuminated into different wavelengths of light if special laser with known wavelength is used by the machine. The light emitted from the labelled cells will be scattered as forward and side patterns. These patterns will be translated into plots by the flow cytometry and graphs could be obtained based on cellular size and granularity. The labelled cells then can be gated and plotted in two new graphs based on the labelling Abs that could discriminate subtypes of cell groups and populations.

2.2.8.1. Surface staining

The labelling protocol was applied to both resting cells and cells post culture with CMV and IL-2 for all the assessed proteins (Table 2.1) according to the following technique:

2.2.8.1.1. HLA-G surface labelling

1. Label 10 FACS tubes with the corresponding cell type.

2. Aliquot 400µl (4×10^5) of fresh/cultured PBMCs in all tubes and double the amount in tubes 8, 9 and 10.
3. Dispense 4µl of anti-human HLA-G APC Monoclonal Antibody (MEM-G/9) (Thermo Fischer Scientific Inc. USA) in tubes 1-5 and 8µl in tube 8.
4. Add 4µl of anti-human CD3 FITC (BioLegend Inc. USA) to tubes 1, 2 and 5.
5. Load 4µl of anti-human CD4 PE, anti-human-CD8 PE, anti-human CD19 PE, anti-human CD14 PE and anti-human CD56 PE (all from BioLegend Inc. USA) into tubes 1, 2, 3, 4 and 5 respectively.
6. Dispense 4µl of anti-human CD69 PerCP-Cy5.5 (eBiosciences Inc. USA) into tubes 1 and 2.
7. Pipette 8µl of anti-human CD4 PE and anti-human CD25 PerCP-Cy5.5 into tube 8.
8. Aliquot 4µl and 8µl of mouse IgG1 Isotype control APC (Thermo Fisher Scientific Inc. USA) in tubes 6 and 9 respectively.
9. Leave tubes 7 and 10 unstained as negative controls.
10. Vortex and incubate at 4° C for 30 min or at RT for 20 min in the dark.
11. By the end of the incubation, dispense 2ml of PBS to all tubes and centrifuge at 400g for 10 min, discard the supernatant and re-suspend the pellets with 0.5ml PBS. The cells were either acquired with the Accuri C6 machine or left in the fridge for a short while until the remaining sets of cells were ready.

2.2.8.1.2. Intracellular staining

The preceding steps included some tubes which were stained with surface markers that were then submitted to intracellular tag staining (tubes 8, 9 and 10) following the company protocol provided in the kit (eBiosciences Inc. USA).

Reagent preparation

- Fixation/Permeabilization Foxp3 working solution (eBiosciences Inc. USA) is prepared by mixing 1:3 volume, based on the number of tubes, concentrated Foxp3 buffer with its diluent fluid respectively.
- Permeabilization working buffer (eBiosciences Inc. USA) is made by diluting 10X stock to 1X, via mixing 1:9 volume with distilled H₂O.

Procedure

1. Following the final step of surface labelling, re-suspend the pellets in tubes 8, 9 and 10 with the residual PBS by pulse vortex.
2. Dispense 1ml of Fix./Per. buffer to all tubes and vortex well.
3. Leave the tubes at 4° C or RT away from light for up to an hour.
4. After incubation, add directly 2ml of permeabilization fluid 1X and centrifuge for 5 min at 500g and decant the supernatant.
5. To the residual pellets, dispense 10µl of anti-human Foxp3 FITC (eBiosciences Inc. USA) to tube 8 and 8µl of Isotype mouse IgG1 FITC (eBiosciences Inc. USA)
6. Incubate the cells for a minimum of 30 min in the dark at RT.
7. Repeat step 4 twice and decant the supernatant at each step.
8. Re-suspend the pellets with 0.5ml PBS; the cells are ready to be analysed with the flow cytometer.

2.2.8.2. KIR2DL4 surface staining

1. Label 3 FACS tubes with the corresponding cell type.
2. Aliquot 400µl (4×10^5 cells) in all tubes.
3. Dispense 4µl of anti-human KIR2DL4 PE (BioLegend Inc. London), anti-human CD3 PerCP-Cy5.5, anti-human CD16 FITC (BioLegend) and anti-human CD56 APC (BioLegend) into tube 1 only.
4. Add 4µl of isotype IgG1 PE (BioLegend) into tube 2.
5. Leave tube 3 as negative control.
6. Vortex and incubate the cells at 4° C for 30 min or at RT for 20 min away from light.
7. By the end of the incubation, dispense 2ml of PBS into all tubes and centrifuge at 400g for 10 min, decant the supernatant and re-suspend the pellets with 0.5ml PBS.
8. The cells were either acquired with Accuri C6 machine or left in the fridge for a short while until the remaining sets of cells were ready.

2.2.8.3. BAFF and BAFF-R surface labelling

1. Label 11 FACS tubes with the matching cell type.
2. Aliquot 400µl (4×10^5) of fresh/cultured PBMCs in all tubes.

3. Add 4µl of anti-human HLA-G APC Monoclonal Antibody (MEM-G/9) (Thermo Fisher Scientific) in tube 1-9.
4. Dispense 4µl of anti-human BAFF FITC (BioLegend) in tubes 1, 3, 5, 7 and 9.
5. Load 4µl of anti-human BAFF-R PerCP-Cy5.5 (eBioscience, Altrincham) into tubes 2, 4, 5, 6 and 8.
6. Dispense 4µl of the following Abs in accordance with the label; anti-human CD4 PE into tubes 1 and 2, anti-human-CD8 PE into tubes 3 and 4, anti-human CD19 PE into tube 5 and anti-human CD56 PE into tubes 8 and 9.
7. Pipette 4µl of anti-human CD14 PE into tubes 6 and 7 (BioLegend) as well as 4µl of anti-human HLA-DR PerCP-Cy5.5 (eBioscience) in tube 6 and anti-human HLA-DR FITC (BioLegend Inc. USA) in tube 7.
8. Add 4µl of mouse isotype IgG1 FITC (BioLegend), 4µl mouse isotype IgG1 APC and mouse isotype IgG1 PerCP-Cy5.5 (eBioscience) into tube 10.
9. Leave tube 11 as negative control.
10. Vortex and incubate at 4° C for 30 min or at RT for 20 min away from light.
11. By the end of the incubation, dispense 2ml of PBS into all tubes and wash at 400g for 10 min, decant the supernatant and re-suspend the pellets with 0.5ml PBS.
12. The cells were either acquired with the Accuri C6 machine or left in the fridge for a short while until the remaining sets of cells were ready.

2.2.8.4. CD103 phenotyping

1. Label 6 FACS tubes with the corresponding cell type.
2. Aliquot 400µl (4×10^5) of fresh/cultured PBMCs in all tubes.
3. Add 4µl of anti-human HLA-G APC Monoclonal Antibody (MEM-G/9) (Thermo Fischer Scientific Inc. USA) in tubes 1-4.
4. Pipette 4µl of anti-human CD103 FITC (BioLegend) into tube 1-4.
5. Dispense 4µl of anti-human CD3 PerCP-Cy5.5 into tubes 1, 2 and 4.
6. Disperse 4µl of anti-human CD4 PE, anti-human-CD8 PE, anti-human CD19 PE and anti-human CD56 PE into tube 1, 2, 3 and 4 respectively.
7. Add 4µl of mouse isotype IgG1 FITC (BioLegend Inc. USA) and mouse IgG1 APC into tube 5.
8. Leave tube 6 as a negative control.
9. Vortex and incubate at 4° C for 30 min or at RT for 20 min away from light.

10. By the end of the incubation, add 2ml of PBS into all tubes and wash at 400g for 10 min, decant the supernatant and re-suspend the pellets with 0.5ml PBS.
11. The cells were either acquired with the Accuri C6 machine or left in the fridge for a short while until the remaining sets of cells were ready.

HLA-G phenotyping			
Tube no.	Cell type	Markers	Label
1	T helper	CD3+CD4+CD69+HLA-G	FITC+PE+PerCPCy5.5+APC
2	T cytotoxic	CD3+CD8+ CD69+HLA-G	FITC+PE+PerCPCy5.5+APC
3	B cell	CD19+HLA-G	PE+APC
4	Monocyte	CD14+HLA-DR+HLA-G	PE+FITC+APC
5	NK cell	CD3+CD56+HLA-G	FITC+PE+APC
6	isotype	Mouse IgG1	APC
7	Negative	-	-
8	Treg	CD4+CD25+Foxp3+HLA-G	PE+PerCPCy5.5+FITC+APC
9	Isotype	Mouse IgG1+Mouse IgG1	APC+FITC
10	Negative	-	-
KIR2DL4 Staining			
Tube no.	Cell type	Markers	Label
1	NK cell	CD3+CD16+CD56+KIR2DL4	PerCPCy5.5+FITC+APC+PE
2	Isotype	Mouse IgG1	PE
3	Negative	-	-
CD103 phenotyping			
Tube no.	Cell type	Markers	Label
1	T helper	CD3+CD4+CD103+HLA-G	PerCPCy5.5+PE+FITC+APC
2	T cytotoxic	CD3+CD8+CD103+HLA-G	PerCPCy5.5+PE+FITC+APC

3	B cell	CD19+CD103+HLA-G	PE +FITC+APC
4	NK cell	CD3+CD56+CD103+HLA-G	PerCPCy5.5+PE+FITC+APC
5	Isotype	Mouse IgG1+Mouse IgG1	APC+FITC
6	Negative	-	-
BAFF and BAFF-R Labelling			
Tube no.	Cell type	Markers	Label
1	T helper	CD3+CD4+BAFF+HLA-G	PerCPCy5.5+PE+FITC+APC
2	T helper	CD3+CD4+BAFF-R+HLA-G	FITC+PE+PerCPCy5.5+APC
3	T cytotoxic	CD3+CD8+BAFF+HLA-G	PerCPCy5.5+PE+FITC+APC
4	T cytotoxic	CD3+CD8+BAFF-4+HLA-G	FITC+PE+PerCPCy5.5+APC
5	B cell	CD19+BAFF+BAFF-R+HLA-G	PE+FITC+PerCPCy5.5+APC
6	Monocyte	CD14+HLA-DR+BAFF+HLA-G	PE+PerCPCy5.5+FITC+APC
7	Monocyte	CD14+HLA-DR+BAFF-R+HLA-G	PE+FITC+PerCPCy5.5+APC
8	NK cell	CD3+CD56+BAFF+HLA-G	PerCPCy5.5+PE+FITC+APC
9	NK cell	CD3+CD56+BAFF-R+HLA-G	FITC+PE+PerCPCy5.5+APC
10	Isotype	Mouse IgG1+Mouse IgG1+Mouse IgG1	APC+FITC+PerCP-Cy5.5
11	Negative	-	-
JEG-3 Labelling			
Tube no.	Cell type	Markers	label
1	JEG-3	HLA-G	APC
2	Isotype	Mouse IgG1	APC
3	Negative	-	-
DC Phenotyping			
Tube no.	Cell type	Markers	Label
1	DC	CD1a+HLA-DR+CD14+HLA-G	PE+FITC+PerCPCy5.5+APC

2	DC	CD86+HLA-DR+CD14+HLA-G	PerCPCy5.5+FITC+PE+APC
3	DC	CD1a+CD86+HLA-DR+HLA-G	PE+PerCPCy5.5+FITC+APC
5	Isotype	Mouse IgG1+	APC
6	Negative	-	-

Table 2.1. Monoclonal Abs used for staining for HLA-G, KIR2DL4 and CD103 as well as BAFF and BAFF-R.

2.2.9. Flow cytometry

Following the labelling steps, cell labelling was assessed by Accuri C6 flow cytometer. The machine setting allows the determination of the required cell number and type of population based on the forward and side scatter pattern that is yielded from cell acquisition. The target count of cells was around 4×10^4 for lymphocytes and 4×10^3 monocytes which can be readily distinguished after rescaling the axes of the main plot. The gates were generated on the main plot and data were presented on another plot revealing the selected sets of cells in addition to the colours that have been allocated for each cell. The machine allows data compensation though real-time acquisition or later after acquisition was completed.

The data generated from the machine were analysed with the software provided by the company (Accuri C6 Plus software, BD Biosciences Inc. USA). The data were plotted on a main graph and then gated for both lymphocytes and monocytes, those gates were transferred to new graphs to be further refined on the scale of cell labelling that can define each group of cells. The isotype and the negative control values were determined and subtracted from the test sample to give the final % positive cells.

2.3. Molecular work

2.3.1. DNA extraction

All healthy as well as renal transplant samples were submitted to DNA extraction following the protocol provided by the company (QIAamp® DNA Mini Kit, Qiagen

Inc. Germany).

Reagent preparation

The kit components Buffer AW1 and AW2 were prepared by adding the required amount of absolute Ethanol (Sigma Aldrich Inc. USA) indicated on each bottle (as instructed in the leaflet provided). All the kit components were brought to RT before proceeding with the protocol steps.

Procedure

1. Dispense 20µl of Proteinase K provided into an Eppendorf tube.
2. Aliquot 200µl of either whole blood or PBMCs at $<5 \times 10^6$ cells/200µl PBS.
3. Pipette 200µl reagent AL, vortex thoroughly to ensure homogenising of the mixture.
4. Leave the tube at 56° C on a hot plate or in a water bath for 10min (Thermo Fisher Scientific).
5. After incubation, vortex the tube and load absolute ethanol 200µl (Sigma Aldrich) to the tube and vortex again to ensure homogenisation, centrifuge the vial to remove any residual attached to the interior of the cap.
6. Load the content of the Eppendorf tube into a new QiaAmp spin column (provided), avoid contaminating the column edges, close the lid and centrifuge at 8000 rpm for 60 seconds.
7. Discard the flow through with the collection tube and place the spin column in to a new collection tube.
8. Disperse 500µl AW1 Buffer into the cartridge column without drenching the edges, close the lid and centrifuge for 60 seconds at 8000 rpm.
9. Repeat step 7.
10. Load 500µl AW2 reagent into the spin column, avoid splashing the rim, put the cap on and centrifuge for 180 seconds at 14000 rpm.
11. Repeat step 7.
12. Spin the column another 60 seconds to dry the cartridge out of AW2 reagent.
13. Repeat step 7.
14. Add up to 200µl PCR water or reagent AE (provided) to the cartridge and leave to sit for 1-5 min and centrifuge for 60 seconds at 8000 rpm.
15. Quantify the harvested DNA with Nano Drop (Thermo Fisher Scientific) and store the sample, if not being used, at 4° C for short term or at -20° C for longer term.

2.3.2. Polymerase chain reaction

2.3.2.1. HLA-G 14bp amplification

The DNA samples produced from the former extraction procedure for both healthy participants in addition to an archived DNA samples obtained from the Royal Liverpool Hospital to establish a link between CMV and rejection episodes following kidney transplantation. Those specimens were analysed for the 14bp insertion/deletion dimorphism of HLA-G in exon 8 of the gene on chromosome 6 short arm at position 6p22.1. Essentially, the primers necessary for the amplification were designated via free commercial software (OligoPerfect™ Designer, Thermo Fisher Scientific Inc. Web Tools). After obtaining the standard nucleotide sequence of the HLA-G molecule (www.ensembl.org), exon 8 was identified and the exact 14bp site was highlighted. The primers were designed to cover 200 nucleotides either side of the 14bp region i.e. the tail region of intron 7 and the mid area of exon 8. The forward and the reverse primers were (5' GTGATGGGCTGTTTAAAGTGTCACC '3) and (3' GGAAGGAATGCAGTTCAGCATGA '5) respectively (Sigma Aldrich). The PCR reaction involved the following components; 100ng DNA, 10mM forward and reverse primers, 8µl ready mix (MyTaq™ Red Mix, Bioline Inc. UK) and 12µl PCR water (Table 2.2). Master mix was prepared dependent on the number of samples included in the experiment in addition to the control and an adequate volume was calibrated to cover 25µl per 200µl Eppendorf tube. All tubes were applied into the thermo cycler machine (Techne Thermal Cycler, Keison International Ltd. UK) and the program set as follow; initial denaturation for 5 min at 94° C, followed by thirty cycles of denaturation at 94° C for 30 seconds, annealing at 63° C for 45 seconds, extension for 60 seconds at 72° C and final extension at 72° C for 8 min (Table 2.3).

By the end of the running time, an agarose gel was prepared by mixing 100ml 1X TMB buffer (Thermo Fischer Scientific Inc. USA) with 2.5g agarose powder (Sigma Aldrich Inc. USA). The mixture was microwaved (Sharp Inc. Japan) to boiling for two min in a 250ml conical flask until no visible clumps or precipitations in the agarose solution were perceived. To allow viewing of the bands, fluorescent dye (Syber Safe, Thermo Fischer Scientific Inc. USA) 1:1000 was dispensed into the liquid agarose straight away after heating as this will permit efficient diffusion of the dye into the gel. The agarose was left on the bench to cool for a short while and poured down into an appropriate electrophoresis tray (StarLab Internationals GmbH).

Germany) with 1mm thickness comb included in the specified slot. The bubbles formed in the gel was pierced with 10µl tip head or dragged to the bottom of the gel to avoid interrupting the bands' migration during the running time. The agarose was left to solidify for approximately 15 min at RT and the comb was removed carefully from the gel to avoid any breaks in the wells formed. The electrophoresis tank (SarLab Internationals GmbH, Germany) was filled to the marked level with 1X TMB running buffer (Thermo Fisher Scientific) and the gel plus the tray were soaked in the running solution to 0.5cm below the TMB surface. The control ladder was prepared following the supplier's instruction (pBR322 *Hae* III Digest, Sigma Aldrich) and the samples generated from the amplification process as well as the negative control and the ladder were loaded carefully (avoid overfilling the wells and stabbing the well's bottom) in each well at 10µl/well. The power was set at 90V and 40mA for 90 min at RT, thereafter, the bands formed after the electrophoresis was detected via a UV transilluminator (BioRad ChemiDoc, BioRad, Hemel Hempstead). The UV machine was set beforehand and the gel was cautiously removed from the electrophoresis tank and positioned in the dedicated aperture, the magnification was refined to deliver the maximum resolution and area of the gel to be photographed.

PCR Components	Sample	Control
DNA	100ng	-
Forward Primer (10Mmol)	1µl	1µl
Reverse Primer (10Mmol)	1µl	1µl
Red Mix 2X	8µl	8µl
PCR water	12µl	12µl

Table 2.2. The master mix components for PCR amplification detection of the 14 bp HLA-G dimorphism.

PCR program	Temperature	Time	
Initial Denaturation	94° C	5 min	
Denaturation	94° C	30 second	30 cycles
Annealing	63° C	45 second	

Extension	72° C	60 second	
Final Extension	72° C	8 min	

Table 2.3. Thermo cycling condition for HLA-G amplification.

2.3.2.2. Single nucleotide polymorphisms (SNP) of exon 8 of HLA-G

The second part of the amplification process involved the identification of SNPs in exon 8 of HLA-G for those healthy individuals and transplant patients who were involved in the study, since these SNPs might be relevant to the level of HLA-G expression. Primers were designed to amplify the 3' end portion of intron 7 up to the 3' end region of exon 8 using commercial software (OligoPerfect™ Designer, Thermo Fischer Scientific Inc. Web Tools). Once exon 8 was defined, the forward and the reverse primers were ordered (Sigma Aldrich), the nucleotide sequences were (5' GTGATGGGCTGTTTAAAGTGTCACC '3) and (3' ACACGTGTACTGTGGAAAGTT '5). The PCR reaction was optimised to a final volume of 25µl/200µl Eppendorf tube including; 100ng DNA, 10mM primers, 8µl ready mix (MyTaq™ Red Mix, Bioline Inc. UK) and 12µl water (Table 2.4). A master mix sufficient for the required samples was set up in accordance with the required number of tests. Afterward, the tubes were allocated in the thermo cycler (Techne Thermal Cycler, Keison International Ltd. UK) and the run was set in the following order; 5 min initial denaturation at 94° C, thirty cycles of 30 seconds denaturation at 94° C, 60 seconds annealing at 59° C and 60 seconds extension at 68° C, this was followed by 8 min final extension at 68° C (Table 2.5). Following the amplification, the PCR products were kept at 4° C until being cleaned up with PCR cleaning kit (Pure Link PCR cleaning, Thermo Fisher Scientific) and were sent off for sequencing (Source Biosciences Inc. UK).

PCR Components	Sample	Control
DNA	100ng	-
Forward Primer (10Mmol)	1.5µl	1.5µl
Reverse Primer (10Mmol)	1.5µl	1.5µl
Red Mix 2X	8µl	8µl
PCR water	12µl	12µl

Table 2.4. The master mix components for exon 8 of HLA-G amplification.

PCR program	Temperature	Time	
Initial Denaturation	94° C	5 min	
Denaturation	94° C	30 seconds	30 cycles
Annealing	59° C	60 seconds	
Extension	68° C	60 seconds	
Final Extension	68° C	8 min	

Table 2.5. Thermo cycling condition for detecting the SNPs of HLA-G.

2.3.2.3 KIR2DL4 genotyping

The HLA-G ligand KIR2DL4 was genotyped to identify the presence of 9 or 10 Adenines in exon 5 of the gene on chromosome 19 long arm at position 19q13.42. DNA samples from healthy individual as well as renal transplant patient recruited in the study were genotyped. The standard sequence of KIR2DL4 was obtained online (www.ensembl.org) and the primers were designed (OligoPerfect™ Designer, Thermo Fisher Scientific Inc. Web Tools) to cover the 3' portion of intron 4 all the way down to the 5' end of intron 5. These were (5' TGCCTGGCAACCAAGAAATG '3) and (3' ACAATCAGGCAACGGTCTGT '5) correspondingly. In 200µl PCR tubes, a total volume of 25µl PCR reaction was added (100ng DNA, 20Mmol primers, 8µl 2X ready mix (MyTaq™ Red Mix, Bioline Inc. UK) and 12µl water (Table 2.6). Master mix was calculated in accordance with the number of samples utilized in each experiment, the tubes were set in a thermocycler (Techne Thermal Cycler, Keison International Ltd. UK) and the program was optimised as following; 95° C initial denaturation for 5 min, then thirty cycles of denaturation at 95° C for 30 seconds, annealing at 59° C for 40 seconds, extension for 1 min at 68° C and a final extension at 68° C for 7 min (Table 2.7).

PCR Components	Sample	Control
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DNA	100ng	-
Forward Primer (20Mmol)	1µl	1µl
Reverse Primer (20Mmol)	1µl	1µl
Red Mix 2X	8µl	8µl
PCR water	12µl	12µl

Table 2.6. The master mix components for KIR2DL4 amplification.

PCR program	Temperature	Time	
Initial Denaturation	95° C	5 min	
Denaturation	95° C	30 seconds	30 cycles
Annealing	59° C	40 seconds	
Extension	68° C	60 seconds	
Final Extension	68° C	7 min	

Table 2.7. Thermo cycling condition for KIR2DL4 amplification.

Agarose gel was set up by mixing 100ml 1X TMB buffer (Thermo Fischer Scientific Inc. USA) with 1.5g agarose powder (Sigma Aldrich Inc. USA). The mixture was heated to boiling for a couple of minutes to allow proper dissolving of the agarose in the TMB buffer. Thereafter, fluorescent dye (Syber Safe, Thermo Fischer Scientific Inc. USA) 1:1000 was added into the hot agarose to allow uniform dispersion of the dye in the gel. The gel was left to cool for a while and dispensed into an electrophoresis tray (StarLab Internationals GmbH. Germany) fitted with a 1mm thickness comb. The gel was let to coagulate at RT and the comb was detached off the gel. Succeeding to this step, the electrophoresis tank (SarLab Internationals GmbH. Germany) was filled to the level with 1X TMB running buffer (Thermo Fischer Scientific Inc. USA) and the gel plus the tray were immersed in the running buffer. The ladder was prepared according to the manufacturer's instructions (50 bp DNA Step Ladder, Sigma Aldrich) and 10µl of the samples generated from the PCR run in addition to the negative control and the ladder were loaded in each well. The

operation power was set at 90V and 40mA for 45 min at RT, later, the bands were spotted by UV transilluminator (BioRad ChemiDoc, BioRad Inc. USA).

2.3.3. Gel extraction

The extraction of the desired band following KIR2DL4 amplification was achieved by location of the bands using the UV transilluminator that is designed for the purpose. Proper protective measures were applied while excising the bands from the gel on the UV transilluminator, the agarose gel being laid over a glass base and the UV light switched on for a minimal period to detect the boundaries of the desired bands and to minimise DNA damage. The selected gel portion was trimmed with a sterile scalpel to remove excess gel because unnecessary extra gel may interfere with the purity of the of the eluted DNA. The gel block was placed in an Eppendorf tube and weighed with and without the gel, as this step is fundamental in the following process. Following the supplier's protocol (PureLink™ Quick Gel Extraction and PCR Purification Combo Kit, Thermo Fisher Scientific) the DNA was prepared to be sent for sequencing.

Reagent preparation

Dispense 10ml Isopropanol (Sigma Aldrich) to reagent B2 and store at RT and aliquot 64ml absolute ethanol to W1 reagent and store at RT.

Procedure

1. Pipette 3:1 volume of L3 reagent into the Eppendorf containing the gel slice.
2. Heat the Eppendorf tube with either a water bath or a heating plate for 10-15 min at 50° C and mix every 2-3 min to assure complete liquefaction and extend the incubation for 5 min after gel melting.
3. Load the melted gel into the centre of a pure link spin column with collection tube provided.
4. Centrifuge the column for 60 seconds at >10000 g and discard the flow through and return in the same collection tube.
5. Pipette 500µl of W1 reagent into the centre of the spin column and centrifuge for 60 seconds at >10000g.
6. Decant the infiltrate and return to the same collection tube.

7. Re-centrifuge at maximum speed for 3 min to remove the wash solution.
8. Discard the collection tube and insert the column into a new Eppendorf tube provided.
9. Dispense 50µl E1 buffer to the spin column and leave it at RT for 60 seconds.
10. Centrifuge the column for 60 seconds at >10000g.
11. Store the eluted DNA at 4° C until ready to be sent for sequencing, if prolonged storage is required, store at -20° C for several months.

2.3.4. PCR product purification

The PCR product yields from exon 8 amplification were directly purified with (PureLink™ Quick Gel Extraction and PCR Purification Combo Kit, Thermo Fisher Scientific) using the same former kit and following the manufacturer's instructions.

Procedure

1. Equilibrate the PCR product to 50µl by using sterile PCR water.
2. Pipette 4:1 volume of B2 buffer to the PCR products and vortex well.
3. Load the sample into the centre of a PureLink spin column with collection tube provided.
4. Centrifuge the column for 60 seconds at 10000g.
5. Decant the infiltrate and return the column into the same collection tube.
6. Dispense 650µl W1 buffer into the centre of the spin column and centrifuge for 60 seconds at 10000g.
7. Discard the flow through and return to the same collection tube.
8. Centrifuge the column once more for 3 min at full speed and discard the collection tube and the infiltrate.
9. Put the spin column in a new recovery tube provided and add 50µl E1 buffer to the centre of the spin column.
10. Allow the tube to sit for 60 seconds and centrifuge again at full speed.
11. The recovered DNA may be stored at 4° C short term or at -20° C for prolonged term.

2.3.5. Sequencing

The cleaned purified PCR products developed from exon 8 amplification in addition to the bands extracted from KIR2DL4 gel were sent off for Sanger sequencing with ABI Big Dye sequencer (Source Biosciences Inc. UK). Online order was made and the samples for exon 8 were coded and packed, the reverse primer (3' ACACGTGTACTGTGGAAAGTT '5) that has been applied for the amplification process mentioned earlier was utilized for sequencing the DNA samples. While, in the case of KIR2DL4, the forward and the reverse primers (5' TGCCTGGCAACCAAGAAATG '3) and (3' ACAATCAGGCAACGGTCTGT '5) that have been applied from the amplification process were sent off with the clean specimens generated from gel extraction steps.

The outcome of the sequencing process was referred electronically to the sender's email address and the data were accessed with some commercial software that allow manipulation and organisation of the sequence samples in comparison with standard sequences.

2.3.6. RNA extraction

The pellets yielded from stimulated and non-stimulated B cells and NK cells isolation were submitted to RNA extraction to assess the HLA-G and KIR2DL4 gene expression on B cells and NK cells respectively. Following the manufacturer's protocol (PureLink® RNA Mini Kit, Thermo Fisher Scientific) the steps were performed on the bench with standard aseptic conditions.

Reagent preparation

- Dispense 60 ml of absolute Ethanol to wash reagent II and mix by inverting up and down.
- Lysis buffer provided is prepared by pipetting 10µl 2-Mercaptoethanol (Sigma Aldrich Inc. USA) with 1 ml lysis buffer, vortexed well.

Procedure

1. Pellet the cells by centrifugation at 2000g for 5 min.
2. Discard the supernatant and add 300µl of lysis buffer with 2-Mercaptoethanol.
3. Vortex thoroughly until the pellets are evenly lysed.

4. Transfer the lysate to a new sterile Eppendorf tube and centrifuge for 2 min at 12000g.
5. Load one equal volume of 70% Ethanol (Sigma Aldrich Inc. USA) to the suspension.
6. Vortex thoroughly.
7. Load up to 700µl of the lysate to the spin cartridge with collection tube provided.
8. Centrifuge the column for 30 seconds at 12000g and discard the flow through and the collection tube.
9. Insert the column in a new collection tube.
10. Pipette 500µl washing buffer II to the spin column.
11. Spin at 12000g for 30 seconds, discard the flow through and the collection tube.
12. Insert the cartridge in a new collection tube and repeat steps 10 and 11.
13. Put the spin column in a new recovery tube and dispense 30-100µl RNase free water to the centre of the spin column.
14. Leave the tube at RT for 60 seconds.
15. Spin the column at maximum speed for 2 min to elute the RNA.
16. Store the eluted RNA at -20° C for short term or at -70° C for longer term use.

2.3.7. cDNA synthesis

The eluted RNA from stimulated and unstimulated B cells and NK cells was quantified using Nano Drop (Thermo Fischer Scientific Inc. USA). The harvested RNA was consumed in the process of cDNA generation according to the supplier instructions (High Capacity cDNA Reverse Transcription Kits, Applied Biosystems Inc. USA). The kit components were ready to use and all steps were performed on the bench (Table 2.8) with high standard aseptic conditions and minimal contamination risk.

Procedure

1. Let the kit compartments thaw on ice, all steps should be executed on ice or freezing rack (StarLab Internationals GmbH. Germany).
2. Prepare a master mix of the reaction to cover the essential number of samples to be converted to cDNA in a final volume of 20µl or above to be adequate for the required assessed probes.

3. Use sterile 200µl Eppendorf tubes, sterile filter tips and new gloves throughout the whole steps.
4. Pipette 4µl of RNase free water into the master mix tube.
5. Add 2µl random primer.
6. Load 2µl RT buffer 10X.
7. Dispense 1µl 10X dNTP to the reaction.
8. Dispense 1µl reverse transcriptase to the mixture.
9. Vortex well.
10. Transfer and mix 10µl of the master with 10µl total RNA at 200ng/µl.
11. Centrifuge briefly.
12. The reaction components were placed in a thermo cycler machine (Techne Thermal Cycler, Keison International Ltd. UK) programmed as one cycle at 37° C for 60 min.
13. By the end of the incubation, proceed to qPCR step or store the tubes at -70 ° C for later processing.

cDNA synthesis	
Component	Volume
Total RNA 200ng/µl	10µl
Random Primers	2µl
dNTP 10X	1µl
Reverse Transcriptase	1µl
RT Buffer 10X	2µl
RNase free water	4µl

Table 2.8. The master mix components for cDNA synthesis.

2.3.8. Real-Time PCR

2.3.8.1. HLA-G gene expression assay

The qPCR experiment was designed to evaluate HLA-G gene expression by stimulated and non-stimulated B cells in response to CMV induction at day 7 of the experiment. The probes were pre-designed to amplify exon 5 and 6 on chromosome

6 short arm of HLA-G region (Assay ID: Hs00365950_g1, Applied Biosystems Inc. USA). The master mix required for the reaction was also ordered from the same supplier (TaqMan® Universal Master Mix II, with UNG, Applied Biosystems) in addition to the control positive housekeeping gene which was appropriate control for blood samples (Assay ID: Hs01087168_m1, Applied Biosystems).

The number of assays were predetermined to cover a duplicate sample including the housekeeping genes. A master mix was prepared and the reaction plate was loaded with cDNA from the stimulated and non-stimulated B cells in addition to the probes and the master mix in a final volume of 25µl per reaction. A standard aseptic technique was maintained throughout the experiment by using sterile filter tips, spraying benches with bleach and changing gloves when possible, the reaction set on a freezing rack (StarLab International GmbH, Germany).

Procedure

1. Excise the required number of wells from a qPCR plate.
2. Dilute each cDNA (from stimulated and non-stimulated B cells) 1:4 with RNase free water and vortex.
3. Aliquot 11.25µl cDNA from stimulated B cells into wells 1-4.
4. Load 11.25µl cDNA from non-stimulated B cells into wells 5-8.
5. Prepare a master mix of the qPCR reaction in a sterile 200µl Eppendorf tube containing [1.25µl HLA-G probe and 12.5µl 2X TaqMan Universal Master Mix II]X4.
6. Formulate another master mix in sterile Eppendorf tube comprising [1.25µl housekeeping gene probe and 12.5 2X TaqMan Universal Master Mix II]X4.
7. Mix well.
8. Dispense 13.75µl HLA-G master mix in wells 1, 2, 5 and 6.
9. Pipette 13.75µl Housekeeping gene master mix in wells 3, 4, 7 and 8.
10. Briefly centrifuge the plate.
11. Load the plate into the qPCR machine (Applied Biosystems 7300 PCR system).
12. Set the qPCR machine to a standard thermo cycling condition (the first stage at 50° C for 2 min, the second stage consists of 50 cycles at 95° C for 10 min and 95° C for 15 seconds followed by a third stage at 60° C for 2 min), label each well with the probe used and adjust the cycle number to 50.
13. Run the machine, it will automatically calculate the time and generates signal graphs for the amplification process in real time for each of the test wells.

2.3.8.2. Cytokines, BAFF and BAFF-R gene expression assay

The assessment of relevant cytokines that were associated with CMV stimulation were accomplished via the use of some available probes at Alder Hey Children's Hospital that were kindly provided by Dr B Flanagan. These probes included (TGF- β , INF- γ , TNF- α , BAFF, BAFF-R, IL-6 and IL-8) which were supplied from (Applied Biosystems Inc. USA) (Table 2.9) also, Master Mix II and HKG that were previously mentioned were utilized in the experiment (table 2.10). A separate master mix was prepared for each probe in a final volume of 25 μ l from stimulated and non-stimulated B cells and loaded in qPCR plate. Standard aseptic techniques were maintained throughout the whole steps to avoid any risk of contamination.

Procedure

1. Determine the number of wells needed to run a duplicate sample including induced and non-induced B cells as well as HKG.
2. Dilute each cDNA (from stimulated and non-stimulated B cells) 1:4 with RNase free water and vortex.
3. Aliquot 11.25 μ l cDNA from stimulated B cells into wells 1-4.
4. Load 11.25 μ l cDNA from non-stimulated B cells into wells 5-8.
5. Prepare a master mix of the qPCR reaction in sterile 200 μ l Eppendorf tube containing [1.25 μ l TGF- β probe and 12.5 μ l 2X TaqMan Universal Master Mix II]X4.
6. Formulate another master mix in a sterile Eppendorf tube comprising [1.25 μ l housekeeping gene probe and 12.5 μ l 2X TaqMan Universal Master Mix II]X4.
7. Mix well.
8. Dispense 13.75 μ l TGF- β master mix in wells 1, 2, 5 and 6.
9. Pipette 13.75 μ l HKG master mix in wells 3, 4, 7 and 8.
10. Repeat step 5, 6, 7, 8 and 9 for each tested probe in the corresponding wells of the qPCR plate.
11. Briefly centrifuge the plate.
12. Load the plate into the qPCR machine (Applied Biosystems 7300 PCR system).
13. Set the qPCR machine to a standard thermo cycling condition (the first stage at 50° C for 2 min, the second stage consists of 50 cycles at 95° C for 10 min and 95° C for 15 seconds followed by a third stage at 60° C for 2 min), label each well with the probe used and adjust the cycle number to 50.

14. Run the machine, it will automatically calculate the time and generates signal graphs for the amplification process in real time for each of the test wells.

Components	B cell induced	Control	B cell non-induced	Control
cDNA	2.81µl	2.81µl	2.81µl	2.81µl
HLA-G Probe	1.25µl	-	1.25µl	-
HKG gene Probe	-	1.25µl	-	1.25µl
TaqMan Master Mix II	12.5µl	12.5µl	12.5µl	12.5µl
RNase free water	8.44µl	8.44µl	8.44µl	8.44µl
Components	B cell induced	Control	B cell non-induced	Control
cDNA	2.81µl	2.81µl	2.81µl	2.81µl
TGF-β Probe	1.25µl	-	1.25µl	-
HKG gene Probe	-	1.25µl	-	1.25µl
TaqMan Master Mix II	12.5µl	12.5µl	12.5µl	12.5µl
RNase free water	8.44µl	8.44µl	8.44µl	8.44µl
Components	B cell induced	Control	B cell non-induced	Control
cDNA	2.81µl	2.81µl	2.81µl	2.81µl
INF-γ Probe	1.25µl	-	1.25µl	-
HKG gene Probe	-	1.25µl	-	1.25µl
TaqMan Master Mix II	12.5µl	12.5µl	12.5µl	12.5µl
RNase free water	8.44µl	8.44µl	8.44µl	8.44µl
Components	B cell induced	Control	B cell non-induced	Control
cDNA	2.81µl	2.81µl	2.81µl	2.81µl
TNF-α Probe	1.25µl	-	1.25µl	-
HKG gene Probe	-	1.25µl	-	1.25µl
TaqMan Master Mix II	12.5µl	12.5µl	12.5µl	12.5µl
RNase free water	8.44µl	8.44µl	8.44µl	8.44µl
Components	B cell induced	Control	B cell non-induced	Control
cDNA	2.81µl	2.81µl	2.81µl	2.81µl
BAFF Probe	1.25µl	-	1.25µl	-
HKG gene Probe	-	1.25µl	-	1.25µl
TaqMan Master Mix II	12.5µl	12.5µl	12.5µl	12.5µl
RNase free water	8.44µl	8.44µl	8.44µl	8.44µl
Components	B cell induced	Control	B cell non-induced	Control

cDNA	2.81µl	2.81µl	2.81µl	2.81µl
BAFF-R	1.25µl	-	1.25µl	-
HKG gene Probe	-	1.25µl	-	1.25µl
TaqMan Master Mix II	12.5µl	12.5µl	12.5µl	12.5µl
RNase free water	8.44µl	8.44µl	8.44µl	8.44µl
Components	B cell induced	Control	B cell non-induced	Control
cDNA	2.81µl	2.81µl	2.81µl	2.81µl
IL-6 Probe	1.25µl	-	1.25µl	-
HKG gene Probe	-	1.25µl	-	1.25µl
TaqMan Master Mix II	12.5µl	12.5µl	12.5µl	12.5µl
RNase free water	8.44µl	8.44µl	8.44µl	8.44µl
Components	B cell induced	Control	B cell non-induced	Control
cDNA	2.81µl	2.81µl	2.81µl	2.81µl
IL-8 Probe	1.25µl	-	1.25µl	-
HKG gene Probe	-	1.25µl	-	1.25µl
TaqMan Master Mix II	12.5µl	12.5µl	12.5µl	12.5µl
RNase free water	8.44µl	8.44µl	8.44µl	8.44µl
Components	B cell induced	Control	B cell non-induced	Control
cDNA	2.81µl	2.81µl	2.81µl	2.81µl
IL-15 Probe	1.25µl	-	1.25µl	-
HKG gene Probe	-	1.25µl	-	1.25µl
TaqMan Master Mix II	12.5µl	12.5µl	12.5µl	12.5µl
RNase free water	8.44µl	8.44µl	8.44µl	8.44µl

Table 2.9. Master mix components of qPCR reaction.

qPCR program	Temperature	Time	
Stage 1	50° C	2 min	
Stage 2	95° C	10 min	50 cycle
	95° C	15 second	
Stage 3	60° C	2 min	

Table 2.10. Standard Thermo cycling condition for Cytokine qPCR amplification.

2.3.8.3. KIR2DL4 gene expression

The experiment was set up to evaluate KIR2DL4 gene expression by stimulated and non-stimulated NK cells stimulated by IL-2 at day 14 of the assay. The probes were pre-designed to amplify exon 2 to 3 of the KIR2DL4 region on chromosome 19. (Assay ID: ID: Hs00427106_m1, Applied Biosystems). The master mix required for the reaction (TaqMan® Universal Master Mix II, with UNG, Applied Biosystems) in addition to the housekeeping gene were provided from the same supplier (Assay ID: Hs01087168_m1, Applied Biosystems) (Table 2.11). A duplicate sample plus the HKG were included in the qPCR plate, and an adequate master mix was prepared to cover the assessed NK cells. A reaction mixture of 25µl per well was set up in a freezing rack and standard aseptic technique was maintained throughout the experiment (Table 2.12).

Procedure

1. Detach the required number of wells out of a qPCR plate.
2. Dilute each cDNA (from stimulated and non-stimulated NK cells) 1:4 with RNase free water and vortex.
3. Aliquot 11.25µl cDNA from stimulated NK cells into wells 1-4.
4. Load 11.25µl cDNA from non-stimulated NK cells into wells 5-8.
5. Prepare a master mix for the qPCR reaction in a sterile 200µl Eppendorf tube containing [1.25µl KIR2DL4 probe and 12.5µl 2X TaqMan Universal Master Mix II]X4.
6. Formulate another master mix in a sterile Eppendorf tube comprising [1.25µl HKG probe and 12.5 2X TaqMan Universal Master Mix II]X4.
7. Mix well.
8. Dispense 13.75µl KIR2DL4 master mix in wells 1, 2, 5 and 6.
9. Pipette 13.75µl HKG master mix in wells 3, 4, 7 and 8.
10. Briefly centrifuge the plate.
11. Allocate the plate in the qPCR machine (Applied Biosystems 7300 PCR system).

12. Set the qPCR machine to a standard thermo cycling condition (the first stage at 50° C for 2 min, the second stage consists of 50 cycles at 95° C for 10 min and 95° C for 15 seconds followed by a third stage at 60° C for 2 min), label each well with the corresponding probe used and adjust the cycle number to 50.
13. Run the machine, it will automatically calculate the time and generates signal graphs for the amplification in real time for each well.

Components	NK cell induced	Control	NK cell non-induced	Control
cDNA	2.81µl	2.81µl	2.81µl	2.81µl
HLA-G Probe	1.25µl	-	1.25µl	-
HKG gene Probe	-	1.25µl	-	1.25µl
TaqMan Master Mix II	12.5µl	12.5µl	12.5µl	12.5µl
RNase free water	8.44µl	8.44µl	8.44µl	8.44µl

Table 2.11. Master mix components of qPCR reaction for KIR2DL4 assay.

qPCR program	Temperature	Time	
Stage 1	50° C	2 min	
Stage 2	95° C	10 min	50 cycles
	95° C	15 seconds	
Stage 3	60° C	2 min	

Table 2.12. Standard Thermo cycling condition for KIR2DL4 qPCR amplification.

2.3.9. CMV ELISA

Healthy participants were screened with anti-human-cytomegalovirus IgG ELISA (Product Number: 40-521-475073, GenWay Biotech, Inc. USA) following the manufacturer's protocol.

Reagent preparation

- Plasma specimens were thawed and diluted 1+100 by mixing 10µl sample with 1000µl sample diluent into an Eppendorf tube and vortexed thoroughly.
- The kit components were brought to room temperature (22° C) for 15 min. as recommended in the booklet, reagents were set up as in the protocol.
- The 96 wells plates provided were designed beforehand to include the blank, controls, cut-off and the diluted plasma in duplicate.
- The remaining wells, if any, were removed from the plate and kept in a sealant pouch provided in the kit at 4° C until further consumption.
- The incubation steps were set either at room temperature or at 37° C.

Procedure

1. Aliquot 100µl of the controls, cut-off as well as the diluted plasma into the corresponding wells.
2. Seal the plate with the foil provided.
3. Leave the plate at 37° C for an hour.
4. After the incubation step, detach the sealant and decant the content of the wells on tissue paper to allow proper disposal.
5. Wash the wells with 300µl diluted washing fluid 3X using an ELISA washing machine (Dynex ELISA Washer, Dynex Technologies, USA) or manually. In case of manual washing, avoid mingling the contents of the wells or overfilling with washing fluid.
6. After the washing step, dry off the wells by blotting the plate on tissue paper several times to ensure that no remaining fluid has been left.
7. Add 100µl conjugate solution (anti-CMV IgG) into the wells apart from the blank.
8. Seal the plate and leave the plate at 22° C for half an hour.
9. Wash again as in step 5.
10. Dispense 100µl of substrate (TMB) in each well.
11. Let the plate stand at 22° C for 15 min in the dark.
12. Add 100µl stop solution to the wells, the blue colour will change to yellow.
13. Read the wells' absorbance at 450-620nm no longer than 30 min later with an ELISA reader (Thermo Electron Corporation, USA).
14. Calculate the mean absorbance of controls, cut-off as well as the samples and subtract the value of the blank from all wells after calculation of the mean. Using the cut-off value, determine the positive and the negative result for each well.

2.3.10. sHLA-G ELISA

The plasma samples that have been obtained during PBMC extraction from both healthy donors and renal transplant patients as well as D+/R- archived sera obtained from renal transplant recipients at the Royal Liverpool Hospital and some culture supernatants with and without CMV stimulation were tested for sHLA-G content using an ELISA (Oxford Biosystems Ltd. UK). Following the supplier's protocol, the below steps were performed, all reagents were brought to RT prior to use and the reagents were prepared fresh at the assay time.

Reagent preparation

- The Master calibrator was provided as lyophilised powder which was prepared by adding 625µl dH₂O to the vial, vortexed and left for 15 min at RT. This dilution give rise to a concentration of 625 Unit/ml, serial dilutions were prepared at 125, 62.5, 31.25, 15.63 and 7.81 Unit/ml in 6 Eppendorf tubes.
- Conjugated solution 100X was prepared at 1:100 dilution via dispensing one volume with 99 volumes of conjugate diluent buffer.
- Wash Buffer 10X was set at 1:10 dilution through mixing 1 part with 9 parts of dH₂O.

Sample preparation

Plasma samples involved in the assay were diluted 1:8 with dilution buffer 2 provided in the kit, while, no dilution was recommended for culture supernatant obtained from CMV induced and control PBMCs at day 7 of culture.

Procedure

1. Dispense 100µl of diluted standard, blank and tested samples in the corresponding wells in duplicate.
2. Seal the plate and incubate at 4° C for 20 hours.
3. Remove that plate out of the fridge and wash 5X with washing buffer using an ELISA washer (Dynex ELISA Washer, Dynex Technologies). Following the last wash, wrap and invert the plate with tissue paper and blot several times until no residual fluid is observed in the wells.
4. Load 100µl conjugate solution to all wells.
5. Leave the plate on an orbital shaker at 300 rpm for an hour at RT.

6. Repeat the washing 5X, dry off the wells by tapping on tissue paper thoroughly.
7. Pipette 100µl substrate solution into each well, incubate in the dark for 25 min at RT.
8. Distribute 100µl of stop solution into all wells and read within 5 min using an ELISA reader (Thermo Electron Corporation).
9. Record the absorbance of the wells at 450nm and 630nm, deduct the reading at 630nm from the reading at 450nm. Calculate the average reading of all wells and subtract the blank value from the remaining measurements. Construct a standard curve by plotting the absorbance values against the standard concentrations on an Excel sheet and choose a polynomial curve or the best fit curve, an equation will be generated which facilitates the estimation of the concentration of unknown samples in Unit/ml. The final values of the samples must be multiplied by the dilution factor as they were diluted in the sample preparation step.

2.3.11. sBAFF ELISA

The same set of samples that were utilised for sHLA-G assessment were further evaluated for sBAFF using an ELISA (product code: ab188391-BAFF (TNFSF13B) SimpleStep ELISA® Kit, ABCAM Inc. UK). Following the supplier's protocol, the steps below were performed; all specimens and standard were run in duplicate and the kit components were brought to RT prior to use and the reagents were prepared fresh at the assay time.

Reagent preparation

- The standard provided in the kit was reconstituted at 40ng/ml by adding 500µl DW to the lyophilised powder, vortexed and left at RT for 15 min. Serial dilution of the stock standard mixed with 25BS sample diluent were prepared at a concentration of 5, 2.5, 1.25, 0.63, 0.31, 0.16 and 0.08ng/ml in 7 Eppendorf tubes.
- Antibody Cocktail was set up by adding 4BI diluent to detector and capture Abs. This was achieved by adding of 600µl the detector and the capture Abs to 4.8ml 4BI diluent in a 5ml Falcon tube and vortexed thoroughly.
- Washing Buffer PT 1X was prepared at 1:10 dilution with DW, the reagent was thoroughly mixed and aliquoted in an ELISA washing bottle.

Sample preparation

Plasma specimens were diluted 1:10 with 25BS sample diluent after optimizing serial dilutions of four samples to choose the optimal concentration that gave rise to an OD within the average range of the standard concentration. No dilution was required for the culture supernatant obtained from CMV stimulated and non-stimulated PBMCs at day 7 of culture.

Procedure

1. Dispense 50µL of standard, blank and samples to the corresponding wells.
2. Load to all wells 50µl Antibody Cocktail.
3. Cover the wells with the film provided and leave the plate on orbital shaker set at 400 rpm for an hour.
4. Decant the well's contents and wash three times with washing buffer using an ELISA washer (Dynex ELISA Washer, Dynex Technologies).
5. Invert the plate and wrap with tissue paper, tap firmly to dry off the wells.
6. Pipette 100µl of substrate TMB to all wells, keep the plate away from light on an orbital shaker set at 400 rpm for 10 min.
7. Aliquot 100µl stopping solution to the wells and gently mix the contents for 1 min.
8. Measure the absorbance of the wells within 15 min at 450nm using an ELISA plate reader (Thermo Electron Corporation).
9. Calculate the average OD of the samples and standard and blank, subtract the blank value from average absorbance of samples and standard. Plot the standard OD against the known concentration of the serial dilution of the standard on an Excel sheet, choose a proper standard curve that fits the curve obtained. An equation will be generated to facilitate measurement of sample concentrations.

2.4. Statistical analysis

The results of this thesis were analysed with Graph Pad Prism version 5 and IMB SPSS Statistics version 23 software, most of the comparisons between groups were performed by Paired and Unpaired t test, one-way ANOVA, linear regression and logistic regression.

CHAPTER THREE RESULTS

HLA-G expression in healthy individuals

HLA-G is a non-classical MHC class I molecule that has been reported to be abundantly expressed in foetal trophoblast under physiological condition (Kovats et al., 1990), this protein has been shown to be found on a low percentage on resting mononuclear and other cells (Lozano et al., 2002). The expression of this receptor has been reported to be upregulated in various conditions including virus infections (Shi et al., 2011), autoimmune disorders (Ben Fredj et al., 2016; Geraghty et al., 1987) and tumours (Mociornita et al., 2013). Due to its tolerogenic properties the current study was designed to test whether HLA-G could be upregulated on PBMCs from healthy subjects of known CMV status in response to laboratory CMV AD169 strain induction and to explore which cell population most likely express this molecule. This could further support the association of HLA-G with viral infection and could elucidate the role played by this molecule in escaping immune surveillance. Thus, healthy subjects of known CMV status were recruited and PBMCs were isolated and stimulated with CMV antigen for 7 days. The proportion of resting HLA-G on PBMCs was recorded and following culture. Data acquired with Accuri C6 flow cytometry and the output was analysed with the software provided.

For the purpose of setting the gates and identifying the proper gates for each population of cells, the acquired cells were gated according to cellular size and granularity. A distinctive lymphocytes and monocytes populations were recognised and these were included in gated and the gated population was further plotted on new graphs with common CD markers and specific subset markers to further recognise the desired cell group. The flow cytometry compensation was then carried out and optimised according to the colour combination applied for each cell population. Next, the amount of protein expressed on a specific cell population was measured according to the percentage of cell displaying this antigen and final proportion was obtained after subtracting the isotype and control negative percentage from the assessed group of cells. Calculation of the final value of HLA-G or other assessed molecules were made and graphs were obtained using either Graph Pad Prism or SPSS software.

3.1. Healthy donors

This section will cover screening of the participants with anti-CMV IgG antibody and HLA-G phenotyping on several PBMC populations extracted from healthy donors of known CMV sero status on resting cells as well as after culture with 1µg/ml CMV

antigen for a week. Also, HLA-G genotyping will be covered, including the 14bp and the SNPs association with surface expression of HLA-G, in addition, sHLA-G assessment in healthy donors as well as HLA-G gene expression will be summarised. The protocol for each technique is explained in Chapter 2.

3.2. CMV screening

Healthy subjects were screened with anti-CMV IgG ELISA (Table 3.1). Out of 36 participants 16 subjects were CMV negative and 20 were CMV positive. Measurements exceeding the OD of the cut off value 0.613 were positive, while any OD below the negative cut off value 0.502 were negative. The mean OD for the positive donors was 2.047 and for those with CMV negative the mean OD was 0.096. Interestingly, OD for the positive subjects was clearly high and no equivocal results were yielded in this group, similarly, for the CMV negative individuals the ODs were well discriminated.

No. of subjects	Cut off	Mean OD	CMV status
20	0.613	2.047	CMV+
16	0.502	0.096	CMV-

Table 3.1. Anti-CMV IgG screening of healthy subjects.

3.3. Anti-HLA-G antibody validation with the JEG-3 cell line

In order to validate the anti-HLA-G Monoclonal Ab, the JEG-3 choriocarcinoma cell line was grown and maintained with complete RPMI media and the cells were labelled with either isotype control or anti-HLA-G antibody (clone MEM-G/9) and others were control non-labelled cells. The cells were acquired with an Accuri C6 flow cytometry and the proportion of HLA-G expressed on the non-labelled JEG-3 cells was 0.68% versus 87.47% on the labelled ones indicating that this mAb can efficiently detect HLA-G on the surface membrane of these cells (Fig. 3.1).

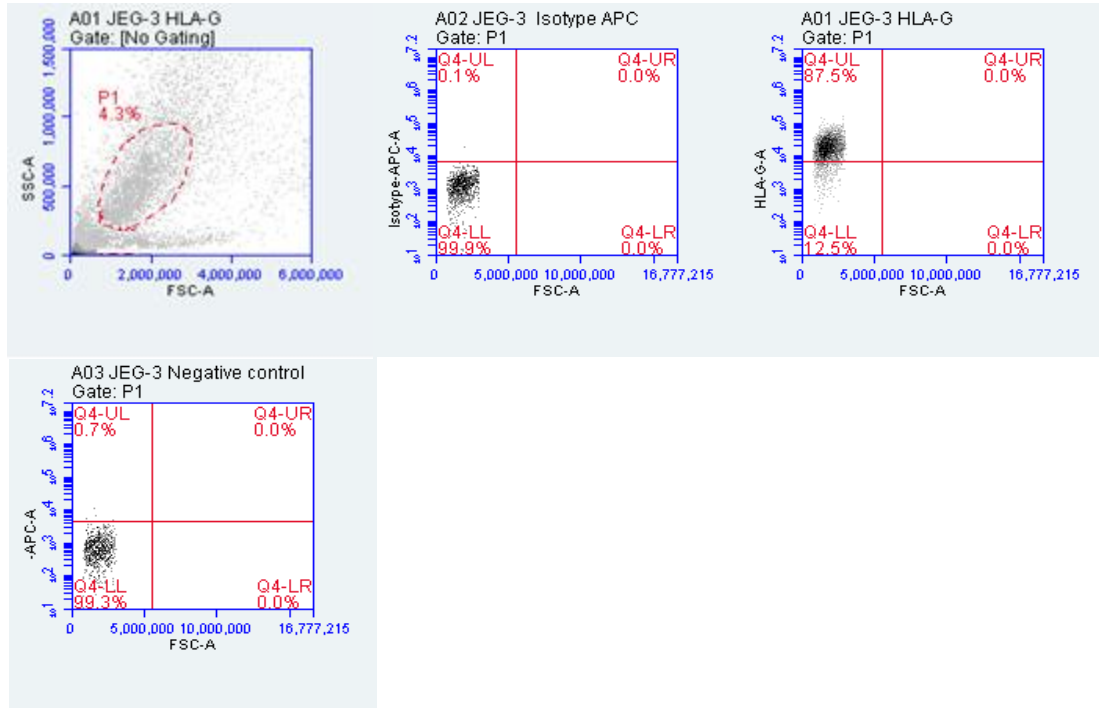


Fig. 3.1. JEG-3 choriocarcinoma cells labelled with anti-HLA-G mAb. Panels show in order: scatter plot showing gated viable cells analysed; the isotype control, anti-HLA-G labelled JEG-3 cells and unlabelled control cells. Figures in the top left quadrant indicate % positive cells.

3.4. Expression of HLA-G on resting PBMCs in CMV+ subjects

HLA-G expression was assessed on the surface of a range of subsets of lymphocytes and monocytes extracted from CMV+ healthy donors (Fig. 3.2). Overall, the population displaying the lowest proportion of HLA-G+ cells was CD3+CD8+ T cells which recorded 0.12% and the highest was CD3+CD56+ lymphocytes at 1.91%. The second-highest expresser was CD19+ lymphocytes with measurement of 1.31% followed by activated CD3+CD4+CD69+ T cells with around 1.03% and 0.51% for monocytes. The other populations of lymphocytes displaying CD3+, CD3-CD56+, CD3+CD8+CD69+, CD4+CD25+FoxP3+, and CD3+CD4+ phenotypes, exhibited low HLA-G percentages, 0.39%, 0.3%, 0.29%, 0.29% and 0.18% respectively.

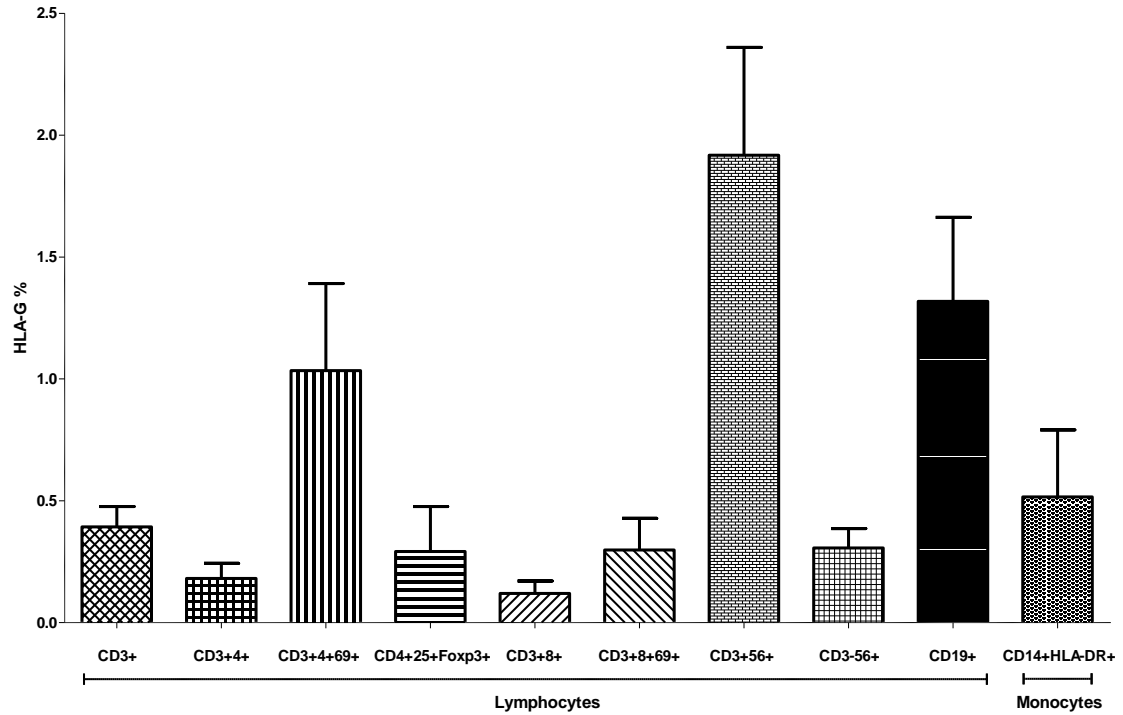


Fig. 3.2. Proportions of HLA-G+ cells for resting PBMC subsets from CMV positive subjects. Results are expressed as mean \pm SEM (n = 20).

3.5. Induction of HLA-G in PBMCs cultured with CMV antigen in CMV+ subjects

After 7 days of cell culture, the proportions of HLA-G+ cells for almost all the studied populations was raised in response to CMV stimulation, particularly, almost 10% of monocytes expressed HLA-G, while Treg which exhibited no HLA-G upregulation in response to the induction scored only 0.24%. These lymphocytes expressing CD3+CD56+, CD3+, CD19+ and CD3+CD4+CD69+ phenotypes, displayed mean proportions of HLA-G+ cells of 7.37%, 0.66%, 6.56% and 2.57% respectively. Proportions of HLA-G+ cells in response to viral stimulation in CD3+CD8+CD69+, CD3, CD56+, CD3+CD4+ and CD3+CD8+ T cells were 1.54%, 1.51%, 0.96%, 0.84% and 0.78% respectively. The highest proportions of HLA-G+ cells were in monocytes as well as CD3+CD56+ lymphocytes (Fig. 3.3). Some representative plots from Accuri C6 flow cytometry are shown in Fig. 3.3a and 3.3b.

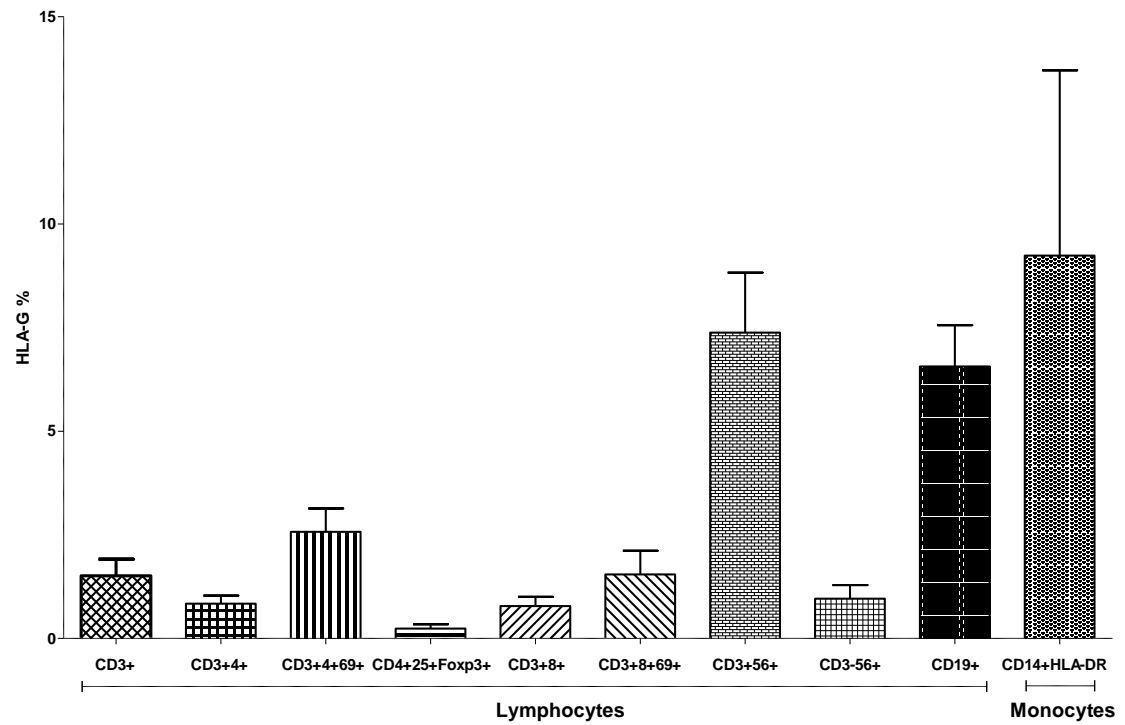


Fig. 3.3. Proportions of HLA-G+ cells for various PBMC subsets on D7 of culture with CMV antigen in CMV+ donors. Results are expressed as mean \pm SEM (n = 20).

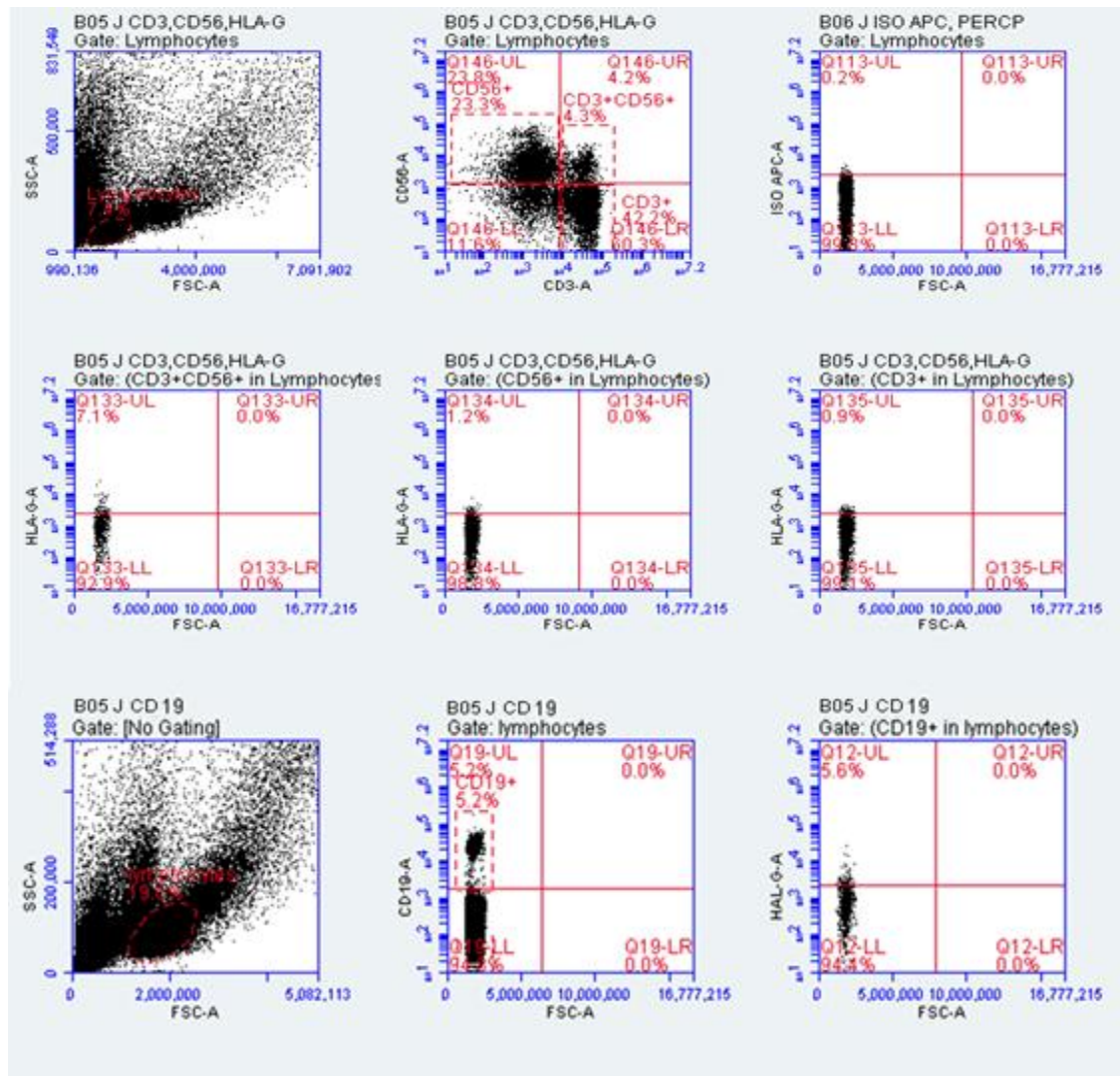


Fig. 3.3a. Representative acquisition plots of Accuri C6 flow cytometry exhibiting in order: scatter profile showing viable lymphocyte gate; CD3 and CD56 co-labeling showing gates used for CD3-CD56+, CD3+ and CD3+CD56+ cells; isotype control; HLA-G proportions on CD3+CD56+, CD3-CD56+ cells and CD3+ cells. Also, the lower panel of plots reveal lymphocytes gating for CD19+ cells and the proportion of HLA-G expressed by these cells.

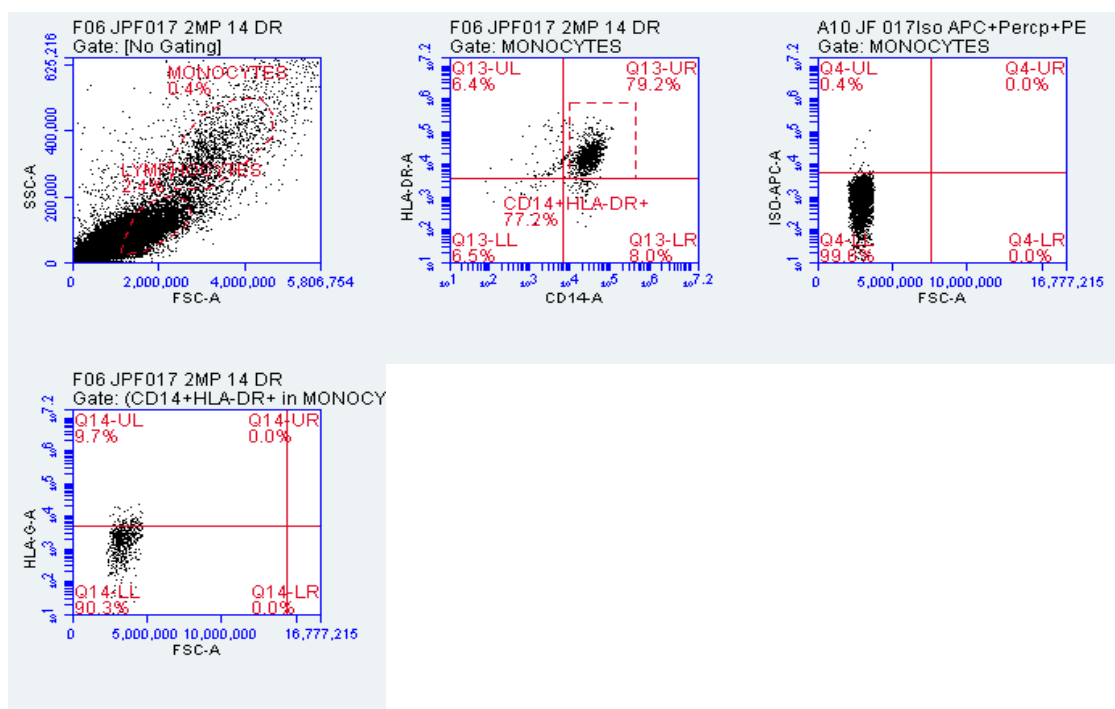


Fig. 3.3b. Representative acquisition plots of Accuri C6 flow cytometry showing in order: scatter profile showing lymphocyte and monocyte gates; CD14 and HLA-DR co-labelling for gated monocytes; isotype control and HLA-G percentage on CD14+HLA-DR+ cells.

3.6. HLA-G expression in CMV+ subjects in resting and cultured PBMCs

The proportions of HLA-G+ cells in various PBMC populations harvested on D7 of culture with CMV antigens were compared to resting proportions of HLA-G+ cells. There were clearly significant differences when a Paired t test two-tailed was performed between these data. CD19+ lymphocytes were revealed to have the highest significant difference with $P=0.0002$, followed by CD3+CD4+ cells with $P=0.0049$, then CD3+CD56+ cells with $P=0.0108$, CD3+CD8+ lymphocytes with $P=0.0148$ and the P value for CD3+CD4+CD69+ cells and CD3+ cells were also below 0.05 ($P=0.0334$ and $P=0.0164$). The remaining subsets of PBMC showed no statistical difference between D0 and D7. However, there was a close to significant difference in CD3+CD8+CD69+ expressing cells at level $P=0.0507$. Indeed, the cell population that showed the most significant difference was CD19 expressing cells (B cells; Fig. 3.4).

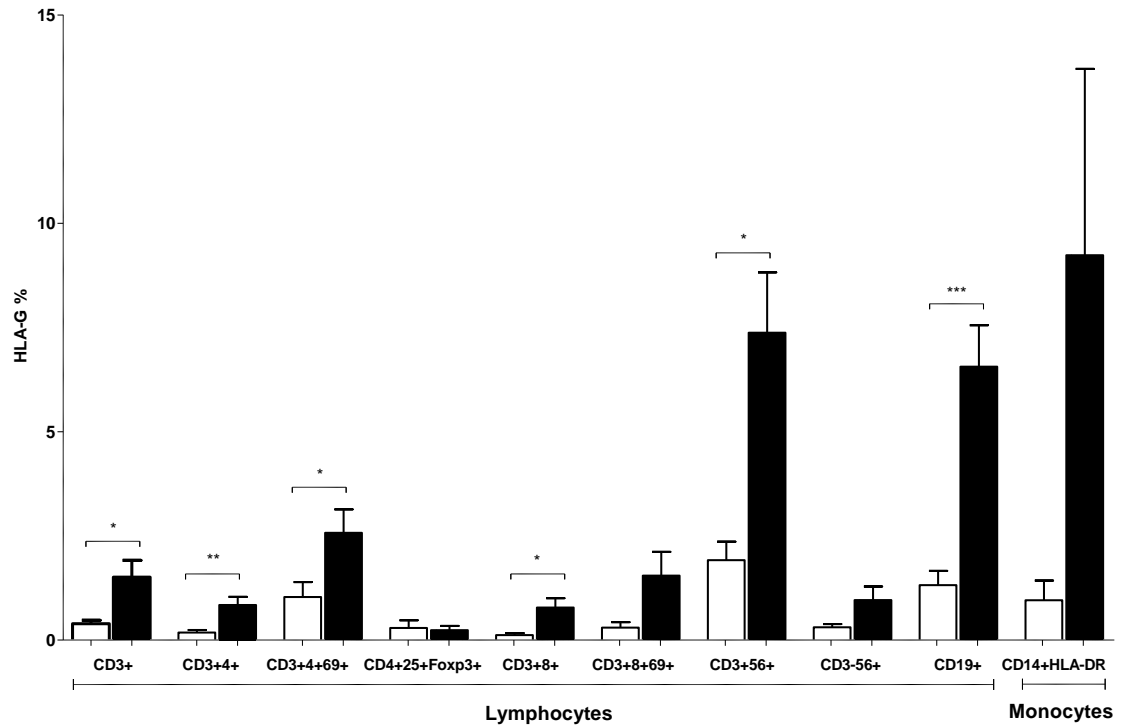


Fig. 3.4. Comparison of proportions of HLA-G+ cells for various PBMC subsets after D0 and D7 of cell culture in CMV+ subjects, empty columns=D0 and filled columns=D7. Results are expressed as mean \pm SEM (n = 20). * $p < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.7. Expression of HLA-G on resting PBMCs from CMV- individuals

Likewise, HLA-G expression was evaluated on the surface of various lymphocyte subsets and monocytes isolated from CMV- donors (Fig. 3.5). Generally, highest proportions of CD3+CD56+ T cells expressed HLA-G at around 3.50%, whereas the least proportion, 0.15%, was found on CD3+CD4+ lymphocytes. Activated CD3+CD4+CD69 lymphocytes ranked the second with a proportion of 2.39% then the monocytes scored 1.51% followed by activated CD3+CD4+CD69+ T cells with 1.5%. The remaining lymphocytes recorded the following levels; CD19+: 0.95%, CD3+CD8+CD69+: 0.80%, CD56+: 0.74% and CD3+CD8+: 0.25%. As with CMV+ subjects, the resting proportions of HLA-G+ cells were highest in CD3+CD56+ cells in CMV- individuals.

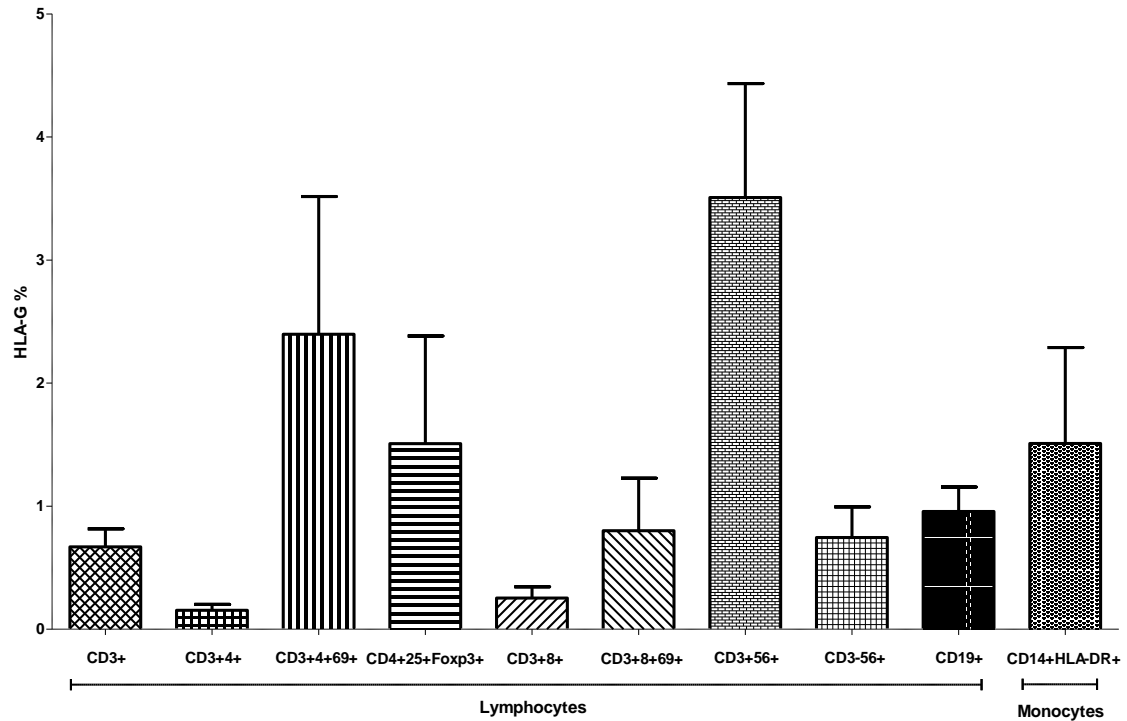


Fig. 3.5. The expression of HLA-G on resting PBMCs isolated from healthy CMV-subjects. Results are expressed as mean +/- SEM (n = 16).

3.8. Induction of HLA-G with CMV antigen in PBMCs from CMV-subjects

The stimulation of PBMCs with CMV antigen as described earlier was performed to assess the difference in comparison to CMV positive healthy individuals. In general, the proportion of HLA-G+ lymphocytes were greatest in CD3+CD56+ T lymphocytes (18.46%) and lowest in T reg (0.41%). The following mean percentages were found on monocytes (8.02%), B lymphocytes (6.70%) and activated T helper cells (4.41%). However, lower proportions of HLA-G+ NK cells, activated cytotoxic T lymphocytes, T helper cells and CD3+CD8+ lymphocytes were found at 1.46%, 1.16%, 0.86% and 0.85% respectively. Strikingly, increased proportions of CD3+CD56+ T cells displayed HLA-G in response to CMV induction (Fig. 3.6).

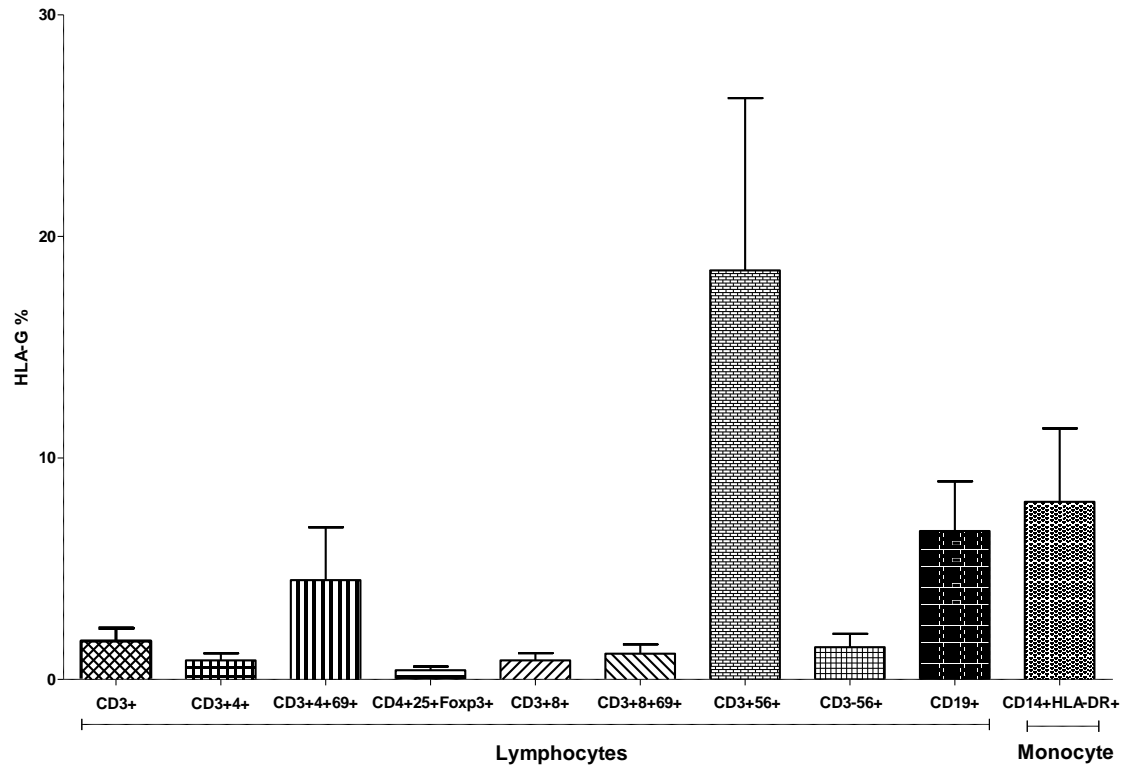


Fig. 3.6. The expression of HLA-G on PBMCs cultured with CMV antigen isolated from healthy CMV - subjects. Results are expressed as mean \pm SEM (n = 16).

3.9. HLA-G expression in CMV- subjects in resting and cultured PBMCs

The expression of HLA-G on almost all stimulated subpopulation of PBMCs was upregulated in response to viral induction apart from T reg which experienced a slight decrease in the proportion of HLA-G+ cells (Fig. 3.7). However, these differences noted on the cultured cells were not statistically significant ($P > 0.05$) when Paired t test, two tailed, was performed and only B cells displayed statistical difference ($P = 0.034$).

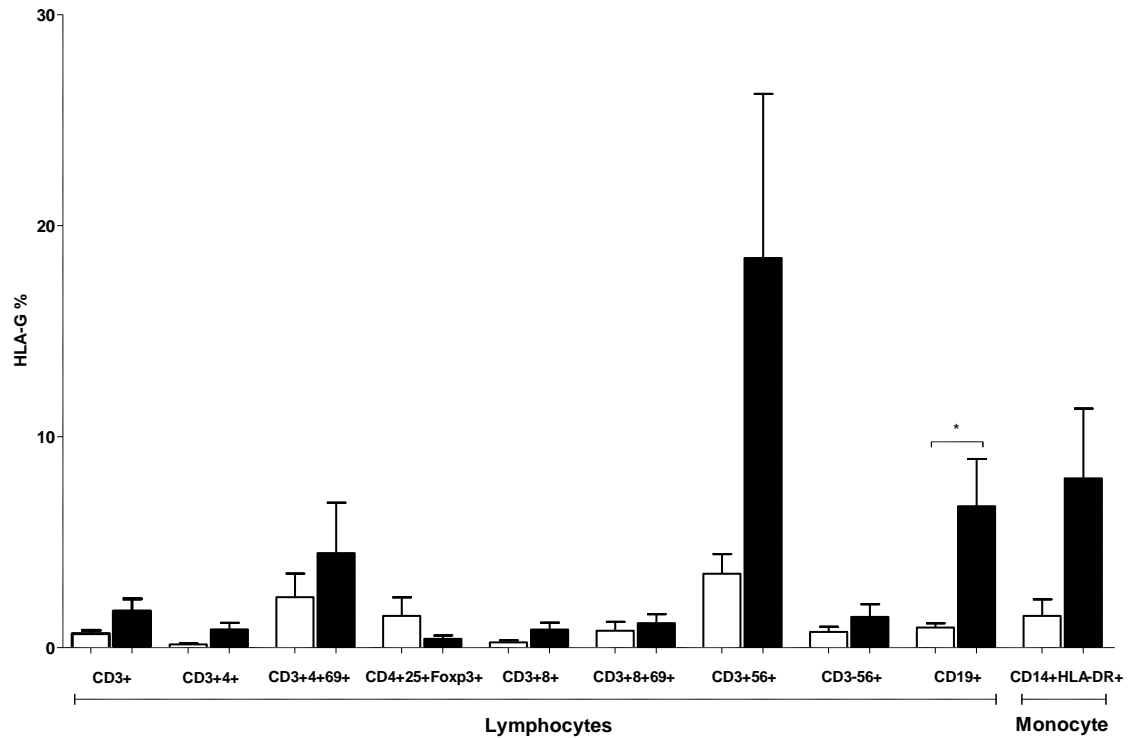


Fig. 3.7. Comparison of HLA-G expression on fresh and cultured PBMCs from healthy CMV- subjects, white columns=D0 and filled columns=D7. Results are expressed as mean \pm SEM (n = 16). * p<0.05.

3.10. HLA-G expression by resting PBMCs in CMV- and CMV+ subjects

Regarding the difference between proportions of HLA-G+ cells on freshly isolated cells from both CMV+ and CMV- groups, there were higher proportions of most subpopulations from CMV- compared to CMV+ subjects except for CD4+ and B lymphocytes which displayed slightly decreased proportions of HLA-G+ cells. However, there were no statistically significant differences between the populations among the tested groups when a two tailed Unpaired t test was applied (Fig. 3.8).

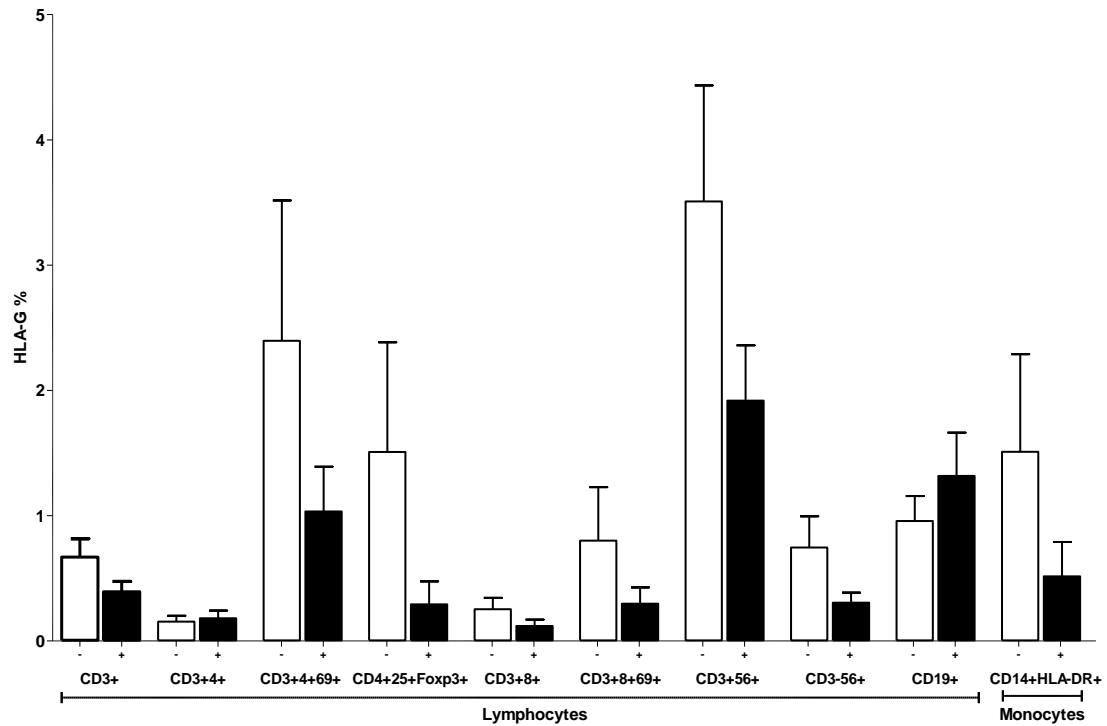


Fig. 3.8. Resting proportions of HLA-G+ cells for various subpopulations of PBMCs from CMV+ (filled columns) and CMV- individuals (empty columns). Results are expressed as mean \pm SEM (n = 20 for CMV+ and 16 for CMV- subjects). None of these differences were significant at the 5% level.

3.11. HLA-G expression in CMV- and CMV+ individuals on D7 of culture

In the same way, to assess which group induced HLA-G more prominently, a comparison was made on PBMCs following culture with CMV antigen from CMV+ and - healthy subjects. As illustrated in Fig. 3.9, the percentage of HLA-G expressing cells on subpopulations from CMV- individuals were slightly higher than the matching populations from CMV+ subjects excluding monocytes and activated CD8+ T cells which showed slightly higher proportions of HLA-G+ cells in CMV+ subjects. However, the only close to significant difference was noted between CD3+CD56+ T cells in the CMV- and CMV+ groups ($P = 0.0773$) when an Unpaired t test (two tailed) was performed. In summary, studies of HLA-G expression in PBMCs from resting and induced cells have revealed consistently higher proportions of positive

cells in CD3+CD56+ T cells, B cells and monocytes from both CMV positive and negative cohorts.

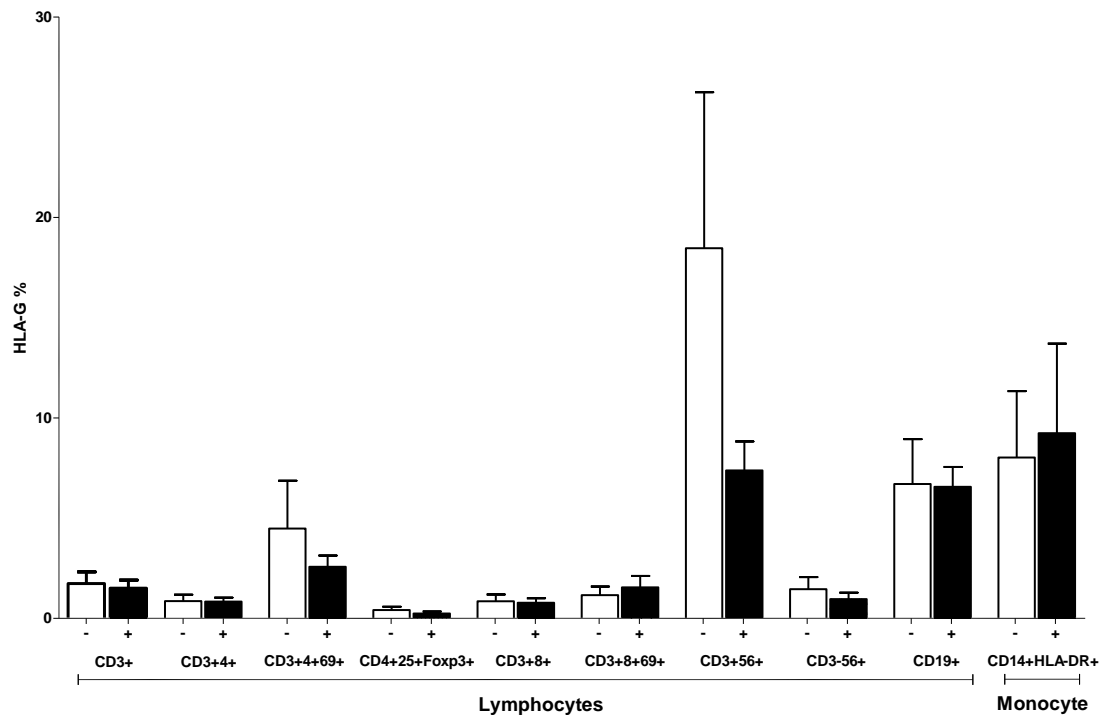


Fig. 3.9. Proportions of HLA-G+ cells in subpopulations of PBMCs post culture with CMV antigen, CMV- (empty columns) and CMV+ subjects (filled columns). Results are expressed as mean \pm SEM (n = 20 for CMV+ and 16 for CMV- subjects). None of these differences were significant at the 5% level.

3.12. HLA-G genotyping

3.12.1. Prevalence of the 14bp dimorphism in exon 8 of HLA-G

It has previously been reported that the presence or absence of a 14bp sequence in exon 8 of the HLA-G gene influences levels of expression (Hviid et al, 2003). The genotyping of HLA-G was performed to test for any correlation between the 14bp insertion/deletion dimorphism and the proportions of HLA-G expressing cells among various PBMC subsets after culture with CMV antigen for a week. Data were acquired either from sequencing or gel electrophoresis of amplified exon 8 that contains the 14bp region. The frequency of insertion/insertion (+/+) homozygotes (41.2%) was the highest among the healthy CMV+ donors and the incidence of insertion/deletion heterozygotes ranked second (35.3%), while deletion/deletion

homozygotes were the least frequent (23.5%). In CMV- individuals, the proportion of heterozygotes (50%) was followed by the homozygous -/- genotype (28.6%) and the least frequent (21.4%) was the +/+ genotype. The χ^2 value for heterogeneity between CMV+ and CMV- donors were not significant (P=0.4967; Table 3.2).

CMV status	+/-	+/+	-/-	Total
CMV+	6 (35.3%)	7 (41.2%)	4 (23.5%)	17 (100%)
CMV -	7 (50.0%)	3 (21.4%)	4 (28.6%)	14 (100%)

Table 3.2. The occurrence of the 14bp HLA-G exon 8 insertion (+) and deletion (-) in healthy CMV+ (n = 17) and CMV- (n = 14) subjects. Differences were not statistically significant (χ^2 test; p=0.4967).

3.12.2. HLA-G expression according to HLA-G genotype

Having established the HLA-G genotype of a panel of healthy subjects, the occurrence of the 14bp insertion/deletion was then tested in relation to HLA-G expression where the proportions of HLA-G+ cells in some PBMC subsets were compared according to genotype through linear regression. No significant correlation (P=0.093) between the frequency of the polymorphic patterns (ins/ins, ins/del and del/del) and proportions of HLA-G+ CD3+CD56+ cells was seen (Table 3.3). Likewise, there was no significant correlation between proportions of HLA-G+ CD19+ B lymphocytes and the occurrence of the 14bp dimorphisms when data were analysed using linear regression (P=0.131). This was also found with monocytes where proportions of HLA-G+ cells showed no significant relation with the 14bp insertion/deletion dimorphism (P=0.224) when the data were submitted to linear regression (Table 3.3).

Linear regression association of HLA-G% on CD3+CD56+ with 14bp	P value
Pearson Correlation Sig. (1-tailed)	0.093
Linear regression association of HLA-G% on CD19+ with 14bp	
Pearson Correlation Sig. (1-tailed)	0.131
Linear regression association of HLA-G% on CD14+HLA-DR+ with 14bp	

Pearson Correlation Sig. (1-tailed)	0.244
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Table 3.3. The association between the expression of HLA-G on CD3+CD56+, CD19+ and CD14+HLA-DR+ cells and the frequency of the 14bp dimorphism, n = 31, P>0.05.

3.12.3. Prevalence of SNPs in exon 8 of the HLA-G gene

In addition to the well-studied 14bp insertion/deletion dimorphism, several SNPs in exon 8 of the HLA-G gene have been associated with high or low expression of the protein. The frequency of the SNPs in exon 8 of the HLA-G gene was evaluated by sequencing in both CMV+ and CMV- subjects. Upon performing a χ^2 test to assess the occurrence of these SNPs among the two groups, the statistical differences between CMV+ and CMV- donors were not significant for all the SNPs and the only SNP that showed close to significant level was C/G +3196 with P= 0.0542 (Table 3.4). In order to quantify the potential influence of exon 8 SNPs on HLA-G expression, a scoring system was developed. Those alleles associated with high HLA-G expression: 14bp del, +3142C and +3187G were allocated a score of 1 while those associated with low HLA-G expression: 14bp ins, +3142G and 3187A were allocated a score of 0. Taking into account both copies of the HLA-G gene, this permitted each healthy subject to be allocated a score of between 0 and 6 indicative of potential for low or high expression. Expression of HLA-G on CD3+CD56+ and CD19+ lymphocytes from healthy subjects at day 7 of cell induction was compared to these scores in Table 3.5 and Figs. 3.10 and 3.10a. Upon comparing the scores in relation to proportions of HLA-G+ CD3+CD56+ lymphocytes in CMV+ and CMV- subjects, no statistical significance was found among the two groups P>0.05 (Fig. 3.10b) when a Mann Whitney test, two tailed, was performed.

Similarly, no significant differences were found (P>0.05) when comparing the scoring with expression of HLA-G by CD19+ cells between CMV+ and CMV- individuals (Fig. 3.10c). In addition, the frequencies of the SNPs were assessed in relation to HLA-G expression on CD3+CD56+ cells (Table 3.6) where linear regression revealed no significant correlation between the SNPs and the proportions of HLA-G+ cells. Likewise, the association between the SNPs and the proportions of HLA-G+ CD19+ lymphocytes was assessed (Linear Regression) and there were statistical associations (P<0.05) with the C/A +3027 and C/T +3035 SNPs (P=0.030 and P=0.022, respectively, Table 3.7), while the C/G +3196 SNP revealed a P value

close to significance ($P=0.059$). The remaining SNPs exhibited no correlation with the proportions of HLA-G+ CD19+ cells ($P>0.05$).

SNPs type		CMV +	CMV -	Total	P value
C/T +3003	C/C	0	0	0	0.5764
	C/T	1	2	3	
	T/T	16	12	28	
G/C +3010	G/G	3	3	6	0.9574
	G/C	8	6	14	
	C/C	6	5	11	
C/A +3027	C/C	12	13	25	0.2761
	C/A	4	1	5	
	A/A	1	0	1	
C/T +3035	C/C	10	12	22	0.2324
	C/T	6	2	8	
	T/T	1	0	1	
C/G +3142	C/C	3	3	6	0.9574
	C/G	8	6	14	
	G/G	6	5	11	
A/G +3187	A/A	8	9	17	0.6285
	A/G	7	4	11	
	G/G	2	1	3	
C/G +3196	C/C	14	6	20	0.0542
	C/G	2	3	5	
	G/G	1	5	6	

Table. 3.4. The frequency of the SNPs in healthy CMV+ and CMV- individuals.

No.	14bp +/-	C/G +3142	A/G +3187	Score	HLA-G expression % on CD3+CD56+	HLA-G expression % on CD19+
1	2	0	1	3	2.32	8.05
2	1	0	0	1	15.02	5.25
3	0	0	1	1	4.32	5.66
4	1	2	1	3	17.67	5.9
5	1	1	0	2	6.88	4.29
6	2	2	2	6	2.54	4.62
7	0	1	0	1	9.96	15.98
8	0	1	0	1	4.76	5.4
9	0	0	0	0	5.1	4.69
10	0	0	1	1	2.4	2.56
11	2	0	1	3	8.7	5.32
12	2	2	2	6	13.55	4.87
13	1	0	1	2	3.86	11.2
14	0	1	0	1	2.69	5.9
15	1	0	1	2	3.89	4.8
16	1	1	0	2	5.61	5.2
17	1	1	0	2	2.85	6.84
18	2	0	0	2	6.21	4.47
19	0	1	0	1	10.82	3.92
20	1	1	0	2	4.11	5.18
21	0	0	0	0	37.58	11.28
22	0	0	0	0	2.7	1.55
23	0	0	1	1	56.8	7.76
24	2	2	2	6	6.01	5.43
25	1	1	0	2	4.36	3.95
26	0	0	1	1	4.68	17.38

27	2	2	1	5	3.46	4.57
28	1	1	0	2	6.18	5.62
29	2	2	0	4	3.92	4.72
30	0	1	1	2	6.84	4.12
31	0	0	1	1	9.83	4.25

Table. 3.5. Scoring the 14bp insertion/deletion polymorphisms (0=+/-, 1=+/+ and 2=-/-) with C/G +3142 (0=G/G, 1=C/G and 2=C/C) and A/G +3187 (0=A/A, 1=A/G and 2=G/G) in a panel of 31 healthy subjects.

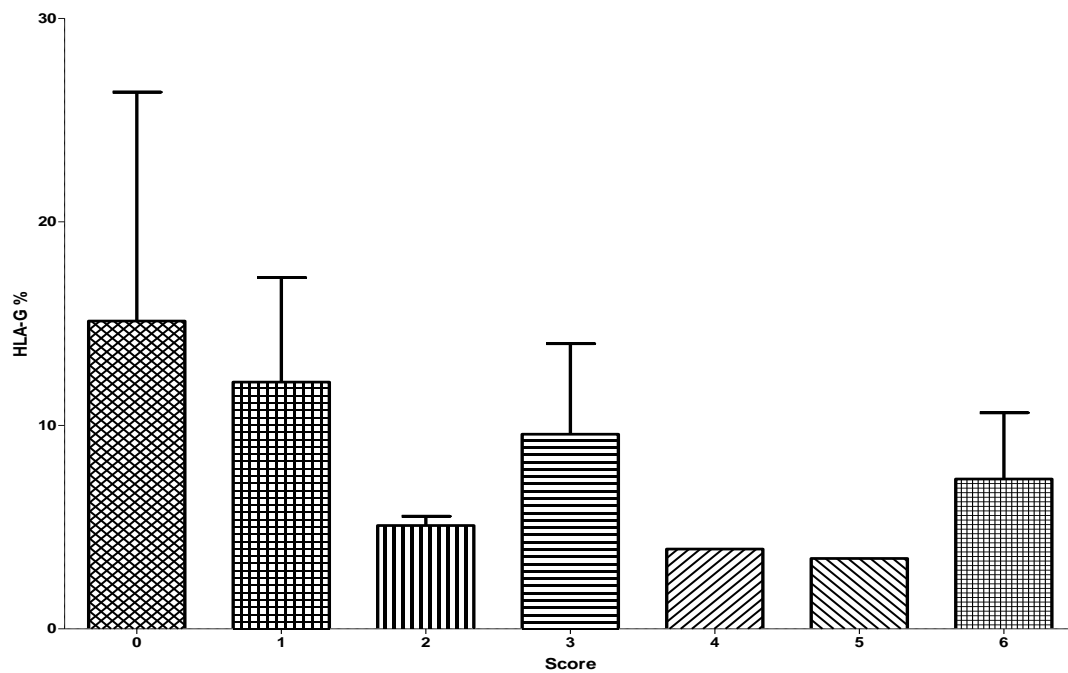


Fig. 3.10. Scoring the HLA-G expression on CD3+CD56+ cells from 31 healthy subjects according to the 14bp insertion/deletion, C/G +3142 and A/G +3178 SNPs. Results are expressed as mean +/- SEM. Columns without error bars indicate a single sample.

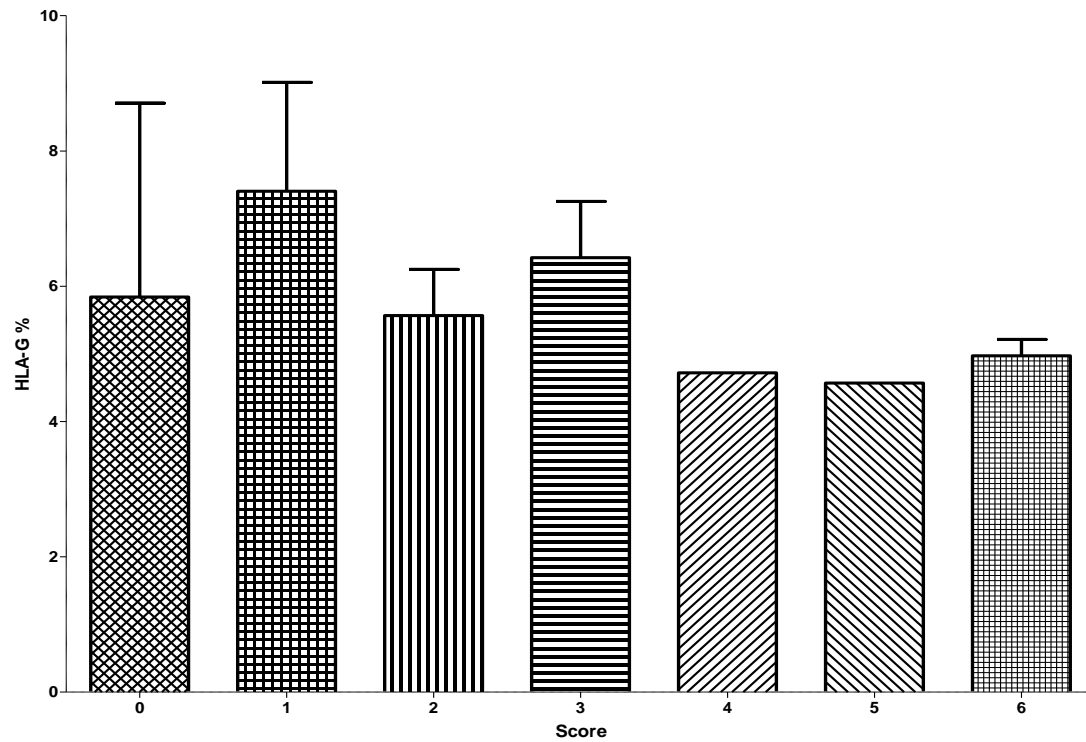


Fig. 3.10a. Scoring the HLA-G expression on CD19+ cells from 31 healthy subjects according to the 14bp insertion/deletion, C/G +3142 and A/G +3178 SNPs. Results are expressed as mean \pm SEM. Columns without error bars indicate a single sample.

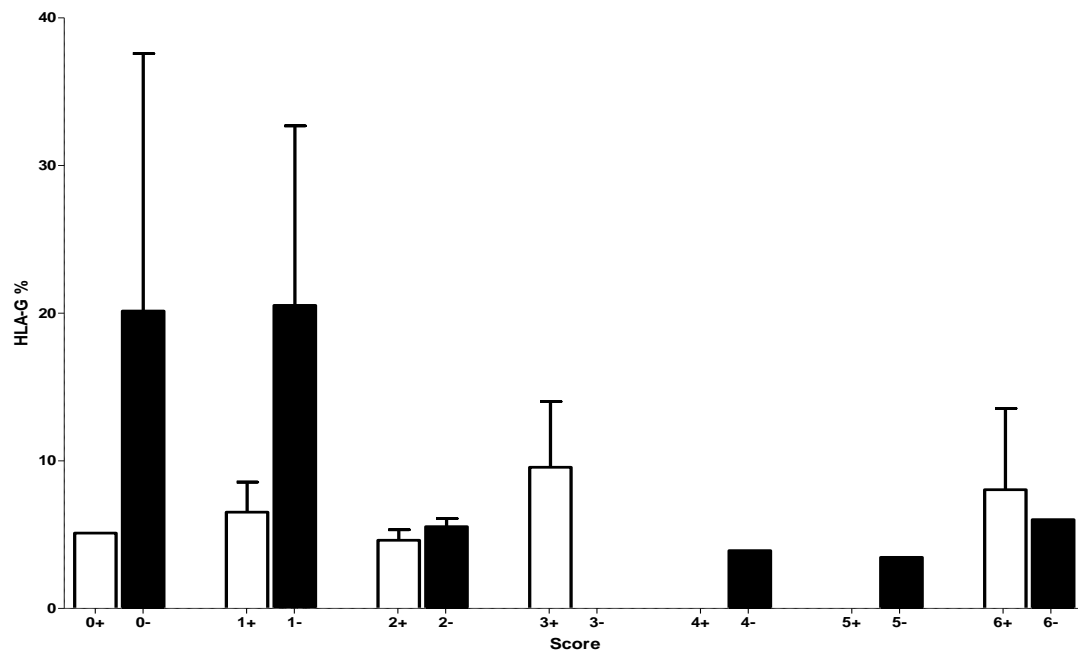


Fig. 3.10b. Comparison of HLA-G expression on CD3+CD56+ cells according to the scoring of the 14bp insertion/deletion, C/G +3142 and A/G +3178 SNPs in CMV+ (empty columns; n = 17) and CMV- healthy subjects (filled columns; n = 14). Results are shown as mean +/- SEM. Columns without error bars indicate a single sample.

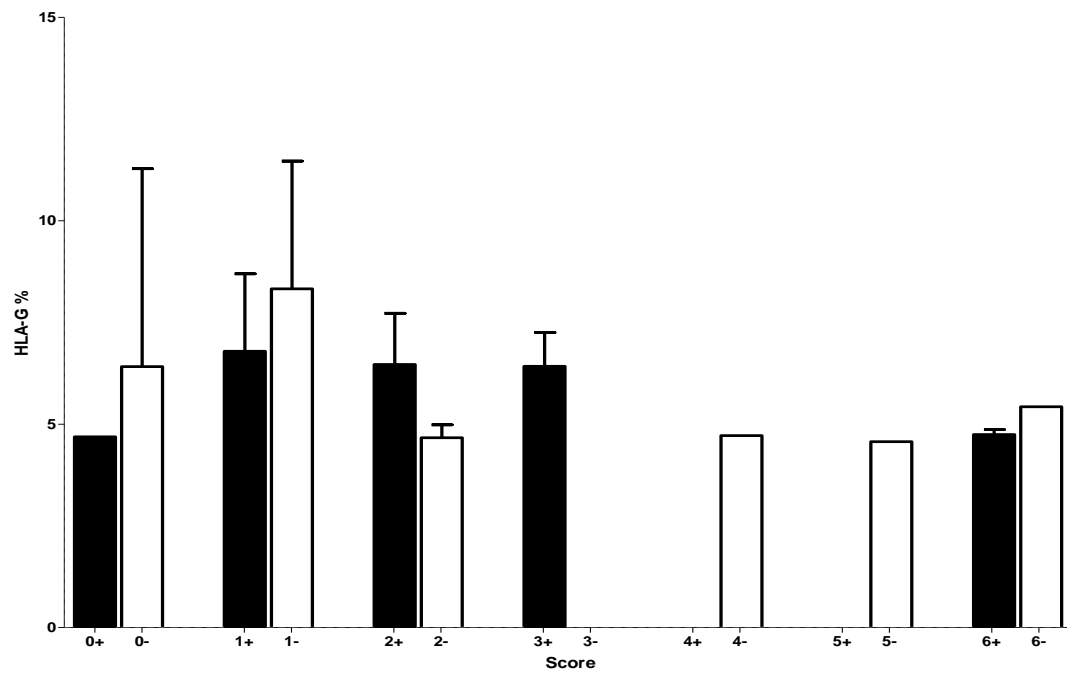


Fig. 3.10c. Comparison of HLA-G expression on CD3+CD56+ cells according to the scoring of the 14bp insertion/deletion, C/G +3142 and A/G +3178 SNPs in CMV+ (empty columns; n = 17) and CMV- healthy subjects (filled columns; n = 14). Results are shown as mean +/- SEM. Columns without error bars indicate a single sample.

Linear regression association of HLA-G% on CD3+CD56+ with C/T+3003		P value
Sig. (1-tailed) Pearson Correlation		0.159
Linear regression association of HLA-G% on CD3+CD56+ with G/C+3010		
Sig. (1-tailed) Pearson Correlation		0.282
Linear regression association of HLA-G% on CD3+CD56+ with C/A+3027		
Sig. (1-tailed) Pearson Correlation		0.163

Linear regression association of HLA-G% on CD3+CD56+ with C/T+3035	
Sig. (1-tailed) Pearson Correlation	0.398
Linear regression association of HLA-G% on CD3+CD56+ with C/G+3142	
Sig. (1-tailed) Pearson Correlation	0.282
Linear regression association of HLA-G% on CD3+CD56+ with A/G+3187	
Sig. (1-tailed) Pearson Correlation	0.398
Linear regression association of HLA-G% on CD3+CD56+ with C/G+3196	
Sig. (1-tailed) Pearson Correlation	0.456

Table 3.6. The association between HLA-G expressed on CD3+CD56+ cells and the SNPs in exon 8 of the HLA-G gene (Linear Regression), n = 31, P>0.05.

Linear regression association of HLA-G% on CD19+ with C/T+3003	P value
Pearson Correlation Sig. (1-tailed)	0.436
Linear regression association of HLA-G% on CD19+ with G/C+3010	
Pearson Correlation Sig. (1-tailed)	0.349
Linear regression association of HLA-G% on CD19+ with C/A+3027	
Pearson Correlation Sig. (1-tailed)	0.03*
Linear regression association of HLA-G% on CD19+ with C/T+3035	
Pearson Correlation Sig. (1-tailed)	0.022*
Linear regression association of HLA-G% on CD19+ with C/G+3142	
Pearson Correlation Sig. (1-tailed)	0.349
Linear regression association of HLA-G% on CD19+ with A/G+3187	

Pearson Correlation Sig. (1-tailed)	0.418
Linear regression association of HLA-G% on CD19+ with C/G+3196	
Pearson Correlation Sig. (1-tailed)	0.059

Table 3.7. The association between HLA-G expressed on CD19+ cells and the SNPs in exon 8 of the HLA-G gene (Linear Regression), n = 31, *P<0.05.

3.13. Soluble HLA-G titres in healthy subjects

Plasma from healthy CMV+ and CMV- individuals and culture supernatants from PBMC from CMV+ and CMV- subjects stimulated with and without CMV antigen were assessed with a sHLA-G ELISA kit (Table 3.8). The supernatant from CMV+ subjects stimulated with CMV antigens displayed significant difference compared to the non-stimulated CMV+ specimens with $P<0.05$ ($P=0.0034$) upon applying a Wilcoxon matched-pairs signed rank test, two tails. However, in the culture supernatant from CMV- subjects, the sHLA-G recorded elevated levels of 2.248 U/ml in the stimulated supernatant versus 1.609 U/ml in the non-stimulated ones and no significant differences was noted when a Paired t test was applied (two tails, P value 0.4290). In the same way, the mean plasma sHLA-G titre in the CMV+ individuals recorded 146.321 U/ml which was greater than the CMV- subjects (78.368 U/ml) with no statistical differences between the two groups upon performing an Unpaired two tails t test ($P=0.5645$).

Serum	CMV-	CMV+	P value
n =	10	13	0.5645
Median (U/ml)	34.2	23.8	
Range (U/ml)	13.5 – 469	17.9 - 1270	
Mean (U/ml)	78.368	146.321	
Culture supernatants, CMV - subjects	Unstimulated	+ CMV antigens	P value
n =	6	6	

Median (U/ml)	1.69	1.02	0.4290
Range (U/ml)	0.04 – 4.49	0.29 – 5.81	
Mean (U/ml)	1.609	2.248	
Culture supernatants, CMV + subjects	Unstimulated	+ CMV antigens	P value
n =	13	13	0.0034
Median (U/ml)	2.41	7.0	
Range (U/ml)	0.175 – 11.2	2.62 – 42.8	
Mean (U/ml)	3.592	21.998	

Table 3.8. Levels of soluble HLA-G (units/ml) in serum and tissue culture supernatants from healthy CMV- and CMV+ subjects cultured for 7 days with or without CMV antigens as measured by ELISA. Statistical analysis was performed using a Wilcoxon matched-pairs signed rank test (two tails).

3.14. HLA-G gene expression

B lymphocytes purified from mixed cultures showed a substantial increase in expression of HLA-G following stimulation with CMV antigens. In order to confirm that this was a result of increased gene expression, B cells isolated from whole PBMCs from 9 healthy CMV+ donors cultured with or without CMV antigen for 7 days were analysed. The qPCR results were calculated based on the relative quantitation of gene expression via the calibration of ΔCT by subtraction of the house keeping gene cycle number from the tested and the control cycles generated by the qPCR machine, Real time PCR curve is displayed in Fig 3. 11a. These values (ΔCT) were then estimated via the equation ($2^{\Delta CT}$) and the measurements were then expressed as percentages. Upon comparing the mean gene expression folds of the non-stimulated HLA-G cells (0.572) with the stimulated B cells (2.532), there was statistical difference between the two groups ($P=0.0039$) when a Wilcoxon matched-pairs signed rank test was applied (Fig. 3.11b).

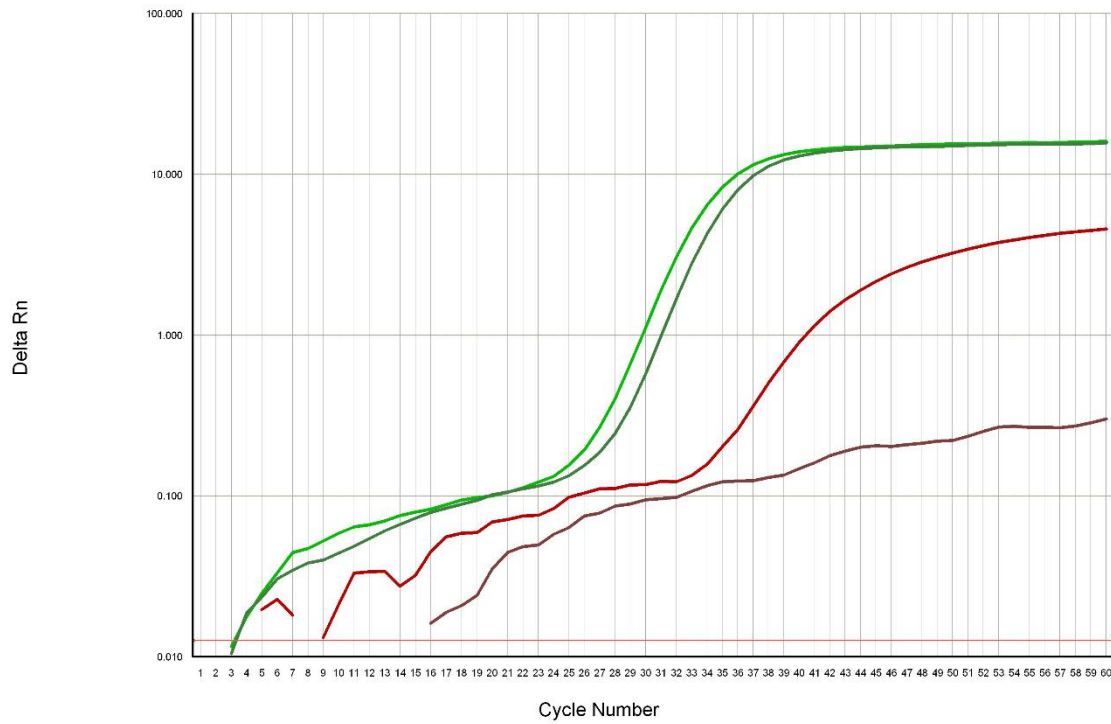


Fig. 3.11a. Real time PCR graph for HLA-G relative gene expression. The left two curves represent the control (the house keeping genes), while the third and the fourth curves emphasise the stimulated and non-stimulated B cells respectively that have been isolated from whole lymphocyte culture.

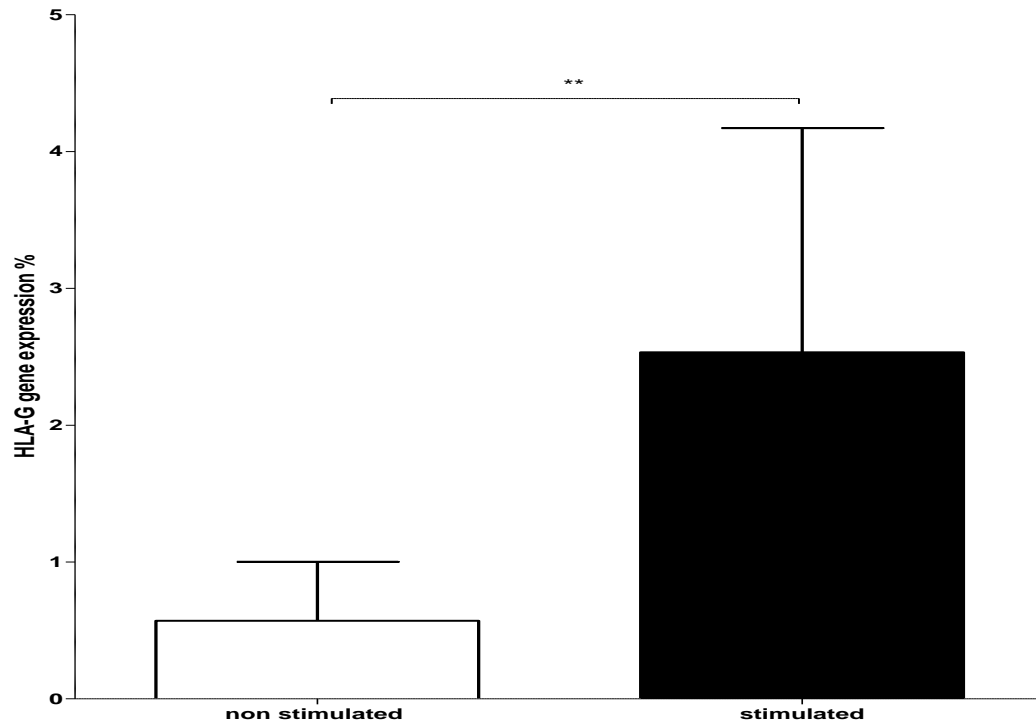


Fig. 3.11b. The relative HLA-G gene expression on B cells isolated from whole PBMCs from CMV+ healthy subjects cultured with and without CMV protein, n = 9. ** P>0.05, Wilcoxon matched pairs signed rank test.

3.15. Summary and discussion of CMV effects on HLA-G expression

Altogether, HLA-G expression in CMV+ subject was significantly upregulated on various cell populations including T helper cells, CTL, B lymphocytes and CD3+CD56+ cells, unlike CMV- subjects were only CD19+ lymphocytes has demonstrated significant HLA-G expression. The frequency of 14bp influence has not shown to correlated with HLA-G increased expression neither with C3+CD56+ nor with CD19+ cells in which both HLA-G was expressed the most. Similarly, SNPs incidence has not shown to correlate with HLA-G expression in CD3+CD56+ but there was positive correlation with occurrence of +3027 and +3035 with HLA-G expressed by CD19+ cells. Likewise, sHLA-G was not found significantly changing in CMV- and CMV+ healthy subjects, yet, culture supernatant from CMV induced PBMCs was significantly higher than control non-stimulated. Real time PCR for HLA-G gene expression was significantly higher in CMV stimulated B cells isolated from whole PBMCs compared to control cells Table 3.9.

Figure	Molecule	Cell population	CMV
3.4	HLA-G	CD3+CD4+	+
3.4	HLA-G	CD3+CD4+CD69+	+
3.4	HLA-G	CD3+CD8+	+
3.4	HLA-G	CD19+	+
3.4	HLA-G	CD3+CD56+	+
3.7	HLA-G	CD19+	-
Table 3.7	A/C+3027	CD19+	Not applicable
Table 3.7	C/T+3035	CD19+	Not applicable
Table 3.8	sHLA-G	PBMCs supernatant	+
3.11	Gene expression	B cells	+

Table 3.9. Summary of significant differences in proportions of HLA-G, SNPs correlation with HLA-G and gene expression of cell subset expression HLA-G in CMV stimulated PBMCs.

The present work findings of HLA-G expression in healthy subjects have shown that cultured PBMCs extracted from CMV- and CMV+ subjects induced with CMV antigens can have significant increase in the proportions of HLA-G expression particularly in CD3+CD56+ and CD19+ cells. However, monocytes also upregulated the protein following culture with CMV antigens but not to significant levels. Onno et al. have shown that the expression HLA-G could be induce in HCMV infection in *in vitro* macrophage culture and the protein was not detected in the non-induced macrophages following incubation for seven weeks (Onno et al., 2000). Monocytes expressed, albeit not to significant levels, suggesting the role of HLA-G in influencing the APC function (Lozano et al., 2002). Another group Yan et al reported a significant increment in the level of HLA-G expression as well as sHLA-G in monocytes and plasma after acute CMV infection (Yan et al, 2009). Since HLA-G can induce tolerance in a variety of pathological and non-pathological circumstances, The upregulation of HLA-G in viral infection can have beneficial effects on the virus enhancing its immune escape from effector cells (Carosella, Moreau, Lemaoult, & Rouas-Freiss, 2008). The upregulation of HLA-G during CMV infection can suppress

and modulate the activity of a variety of HLA-G expressing cells that involve in viral clearance; NK cells have critical effects in inhibiting viral propagation and restricting its spread in active infection and the upregulation of this protein on such cells can drastically suppress such function (Wilkinson et al., 2008). CD56+ T cells in particular have been reported to be increase in healthy CMV+ subjects and they proliferate further following CMV induction as reported by (Almehmadi et al, 2014). This is in accordance with the current study in which significant proportions of CD3+CD56+ cells exhibited HLA-G following CMV induction, suggesting the important role played by these cells in defence against CMV. However, this may also increase their immunosuppressive potentials toward any other host cells expressing HLA-G ligands.

Regarding the HLA-G 14bp polymorphisms, the data here demonstrated no association between the HLA-G protein levels expressed by PBMCs from healthy subjects and the occurrence of the polymorphic patterns (-/-, -/+ and +/+). Previously, unlike other studies where these polymorphisms have associated with either high or low expression of HLA-G molecule. These conflicting results obtained from several groups and the present study could be due to further allelic variations in the HLA-G genes in different populations because some HLA-G alleles i.e. HLA-G*01:01:01 have higher levels of sHLA-G secreted in plasma than G*01:05 N and G*01:01:03, while the former alleles express less sHLA-G than G*01:04:01 (Rebmann et al., 2001). The occurrence of the 14bp insertion polymorphism in exon 8 of HLA-G could lead to less sHLA-G secreted in sera due to lower mRNA quantities (Hviid et al., 2003; Hviid et al., 2004). Added to this, the experiments performed here utilised antigens from a laboratory CMV strain (AD169) to stimulate PBMCs, these viral antigens are missing few gene products found in clinical strains (Cha et al., 1996); as such, it is suggested that laboratory CMV strains do not fully mimic clinical isolates and less extent of effects could be obtained on the studied cell population (Albayati et al., 2017).

Likewise, data from SNPs association with HLA-G expression on some cell populations have shown no clear association between most of the SNPs and the levels of HLA-G+ cells after CMV culture, however, only C/A+3027 and C/T +3035 were positively correlated with increased proportions of HLA-G+ cells after CMV induction. This could be attributed to complex processes involved in the molecular pathways to synthesize HLA-G that could be influence by the promoter region,

degradation and transcription of mRNA, added to this, other polymorphic sites in HLA-G encoding regions can affect the levels of mRNA translation and consequently the amount of HLA-G expression on cell surface or secreted in plasma (E. A. Donadi et al., 2011).

The current study reported no significant differences in sHLA-G concentration in plasma from CMV- and CMV+ individuals as well as from supernatant recovered from CMV- PBMCs culture induced with and without CMV proteins while, supernatant obtained from CMV+ subjects stimulated with or without CMV antigens had a significant increase in sHLA-G after induction. In CMV+ subjects the elevated secretion of sHLA-G in response to CMV induction could indicate the possibility of this protein to interact with receptors for HLA-G like KIR2DL4, ILT-2 and ILT-4 that mediate inhibitory behaviour of some immune cells in the presence of CMV infection (Albayati et al., 2017). Also, CMV+ subjects could have more sHLA-G as those individuals have been exposed to CMV and their response to CMV induction can yield more sHLA-G than non-exposed (CMV-) subjects.

CHAPTER FOUR

RESULTS

**Influence of CMV on KIR2DL4 expression in
healthy subjects**

KIR2DL4 is one of the primary ligands for HLA-G and is expressed mainly by NK cells and a subset of T cells (Rajagopalan & Long, 1999). The receptor has potent immunoregulatory behaviour of either inhibiting or upregulating other immune cells (Vilches & Parham, 2002). Since it can ligate with HLA-G, an evaluation of the role played by this protein was established on the basis of CMV status and according to the occurrence of 9A and 10A alleles in the KIR2DL4 gene which have been reported to influence its expression in NK cells (J. P. Goodridge et al., 2003). In this section, the expression of KIR2DL4 on resting and IL-2 cultured NK cells and lymphocytes was assessed, PBMCs extracted from 22 healthy subjects of known CMV status were labelled with mAbs to measure the proportions of KIR2DL4 expressing resting NK cells and other lymphocytes and after IL-2 culture for two weeks. Also, the occurrence of the 9A and 10A genotypes of the KIR2DL4 gene was identified by sequencing and data were used to classify those healthy individuals as 9A or 10A in order to indicate any influence on surface KIR2DL4 protein expression. Protein expression of KIR2DL4 was detected using mAbs and Accuri C6 flow cytometry and the data obtained were analysed with the software provided. In addition, KIR2DL4 gene expression was assessed in NK cells isolated from whole PBMCs to indicate the mRNA levels after cell induction with IL-2.

4.1. KIR2DL4 expression on resting lymphocytes in CMV+ subjects

The resting KIR2DL4 level was assessed on lymphocyte subsets extracted from 15 CMV+ participants. Fresh PBMC were labelled with anti-CD3, CD16, CD56 and KIR2DL4 monoclonal Abs and data acquired with Accuri C6 flow cytometry, some representative plots for KIR2DL4 on various lymphocytes subsets are shown in (Fig. 4.1a). The highest KIR2DL4 expressers were CD56^{bright} NK cells (CD56+CD16-) of which 10.2% were positive and the lowest expressers were CD3+CD56- cells (T cells; 4.0%). The percentage of NK cells (CD16+CD56^{dim}) expressing KIR2DL4 was 9.2%, followed by CD56+CD3- cells and CD3+CD56+ lymphocytes which scored 9.05% and 6.08% respectively. The CD56+CD3- population includes both CD56^{bright} and CD16+CD56^{dim} populations (Fig. 4.1b).

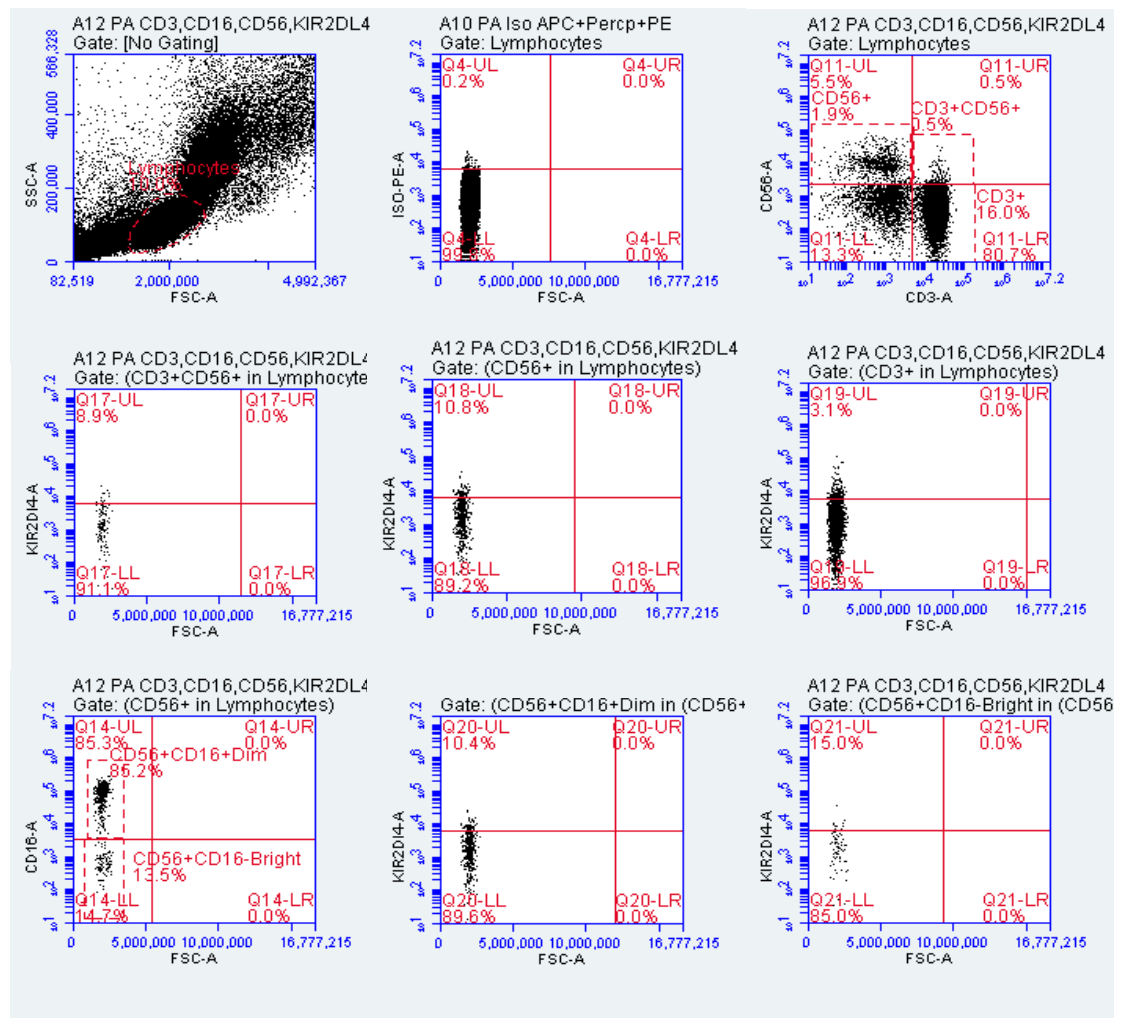


Fig. 4.1a. Representative acquisition plots of Accuri C6 flow cytometry exhibiting in order: scatter profile showing lymphocyte gate; CD3 and CD56 co-labelling showing gates used for CD3-CD56+, CD3+ and CD3+CD56+ cells; isotype control; KIR2DL4 proportions on CD3+CD56+, CD3-CD56+ cells and CD3+ cells. In addition to CD56^{Dim} and CD56^{Bright} expressing the proportion of KIR2DL4 on resting day.

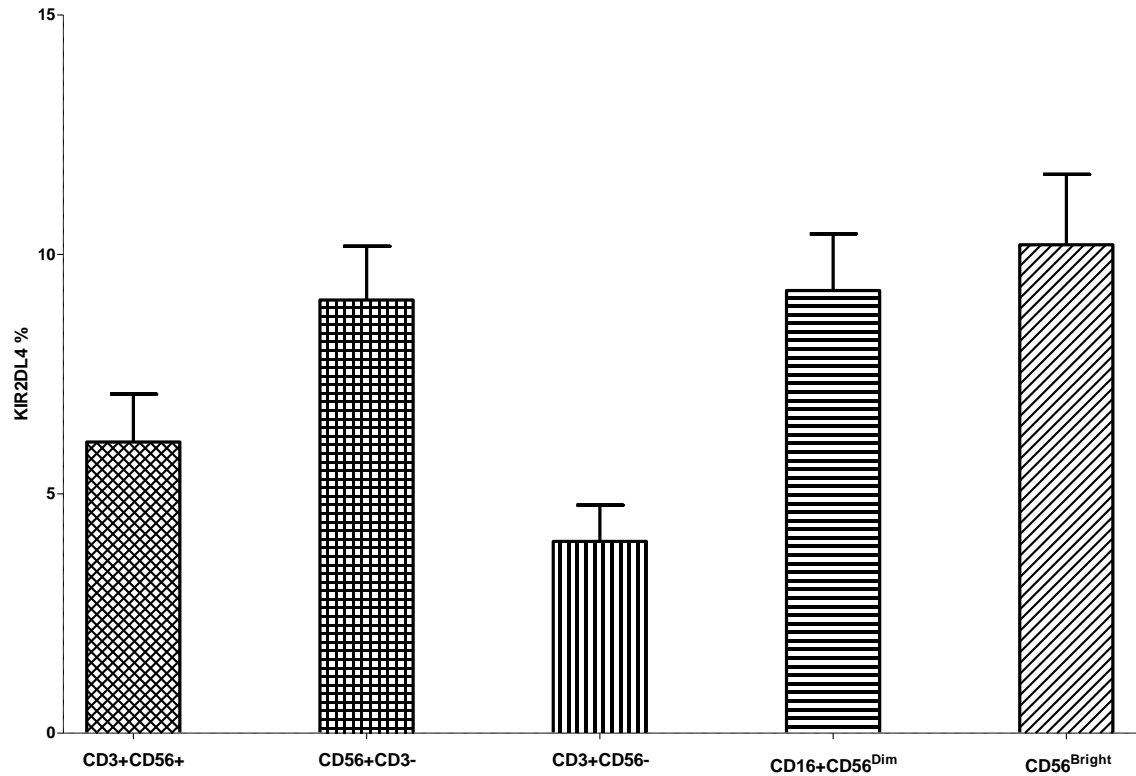


Fig. 4.1b. KIR2DL4 expression on freshly isolated NK cells and other lymphocytes in CMV+ individuals. Results are expressed as mean \pm SEM (n = 15).

4.2. Induction of KIR2DL4 in NK cells and other lymphocytes in CMV+ individuals with IL-2

Following culture with IL-2, KIR2DL4 expression on NK and non-NK cells was measured with Accuri C6 flow cytometry. The cell population with the highest proportion of KIR2DL4+ cells was CD3+CD56+ lymphocytes (12.13%), while only 2.15% of CD3+CD56- cells were positive. The second highest proportion of KIR2DL4+ cells was NK dim cells (CD16+CD56^{dim}) of which 8.44% were positive, while for CD56+CD3- and NK bright cells (CD56^{bright}CD16-) 7.6% and 5.0% of cells were positive, respectively (Fig. 4.2).

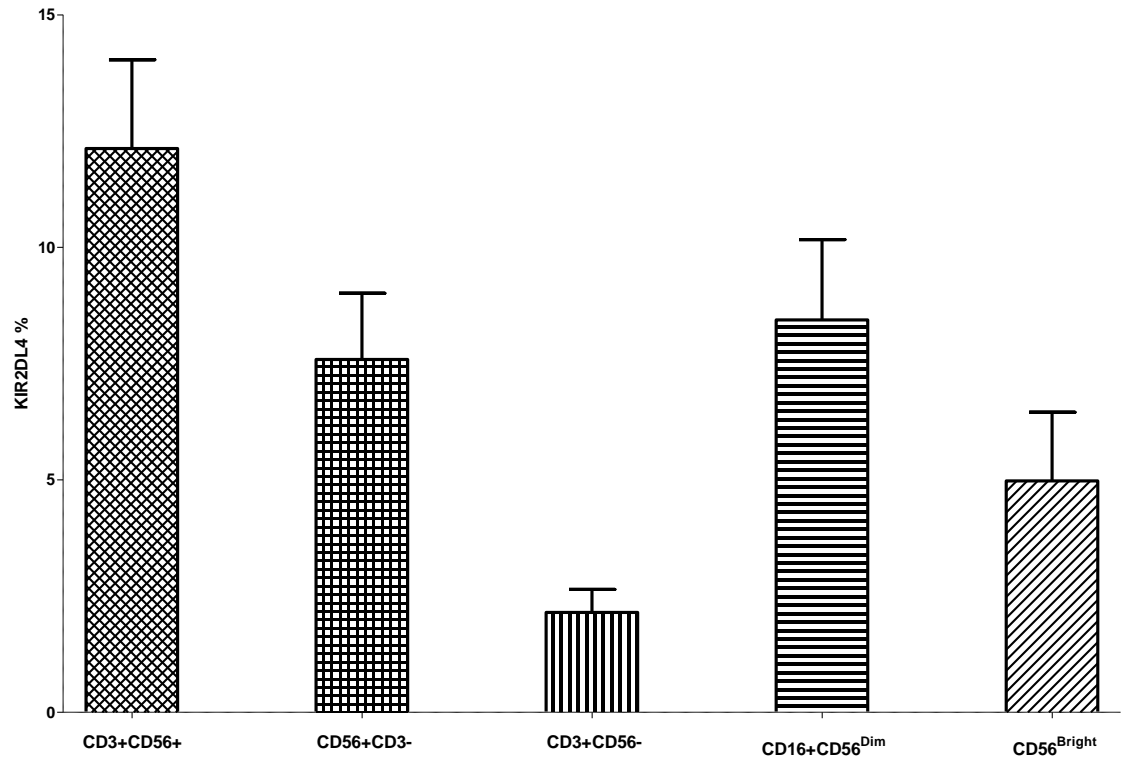


Fig. 4.2. The KIR2DL4 expression on NK cells and other lymphocytes after two weeks of IL-2 induction in CMV + subjects. Results are expressed as mean \pm SEM (n = 15).

4.3. Comparison of KIR2DL4 expression in CMV + subjects on resting cells and following IL-2 induction

Upon comparing the levels of the KIR2DL4 on resting NK and non-NK cells and following stimulation with IL-2 for two weeks in CMV + donors (Fig. 4.3), when a two-tailed Paired t test was applied, CD3+CD56+ cells revealed significant upregulation of KIR2DL4 on D14 of culture ($P=0.0120$). In the same way, NK bright cells (CD56^{bright}CD16-) illustrated significant downregulation of KIR2DL4 after IL-2 culture ($P=0.0195$). The remaining populations (CD56+CD3-, CD3+CD56- and CD56^{dim} NK cells) displayed a consistent pattern of protein downregulation on D14 of cell culture, however, there were no statistical differences between values before and after culture in these cell populations ($P>0.05$) and the P value was close to significant in CD3+CD56+ group $P= 0.0542$. Generally, most of the tested lymphocytes illustrated downregulation patterns of the proteins after cell culture with IL-2.

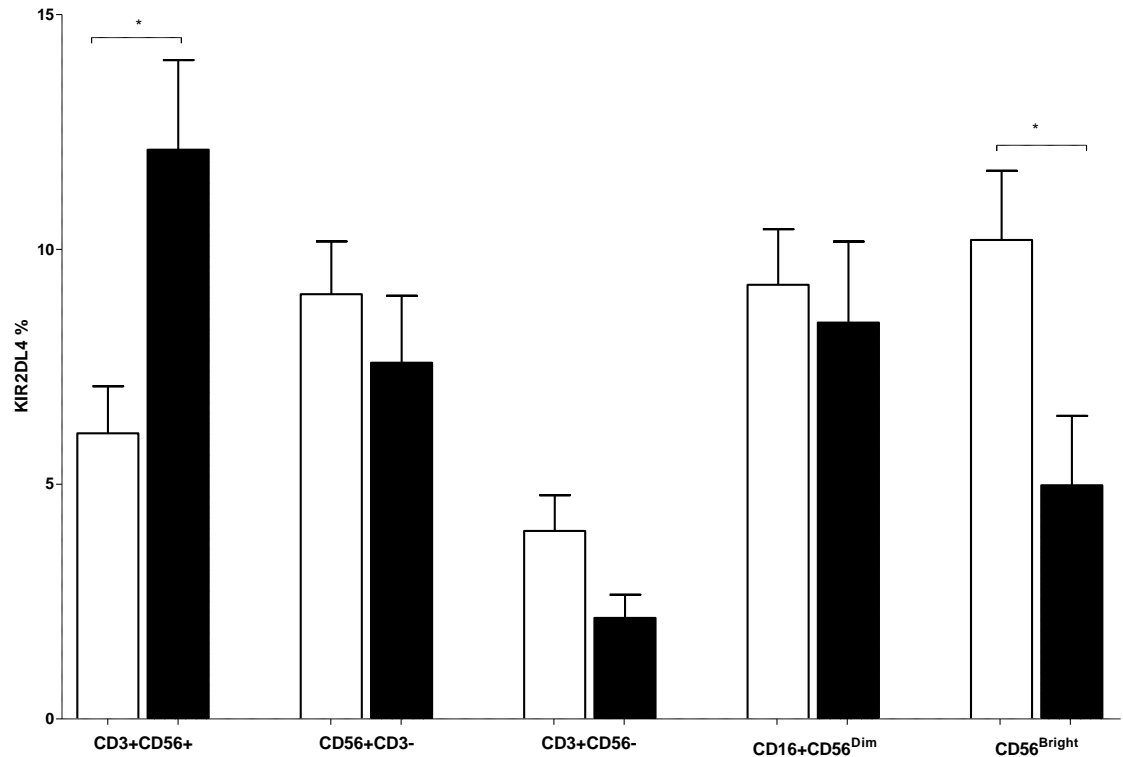


Fig. 4.3. KIR2DL4 expression on resting cells (empty columns) and two weeks after IL-2 culture (filled columns) in CMV+ subjects. Results are expressed as mean \pm SEM (n = 15). * p<0.05.

4.4. Expression of KIR2DL4 on resting lymphocytes in CMV- subjects

Likewise, PBMCs extracted from 7 CMV- donors were evaluated for the expression of KIR2DL4 on NK and non-NK cells and as with CMV+ subjects the cell population with the greatest proportion of positive cells was CD56^{bright} NK cells (CD56^{bright}CD16-) with 7.96% while CD3+CD56- cells had a very low level of KIR2DL4+ cells (2.35%). The other cell populations, CD56+CD3-, CD3+CD56+ and CD56^{dim} NK cells, showed 7.5%, 6.5% and 5.9% of KIR2DL4+ cells, respectively (Fig. 4.4).

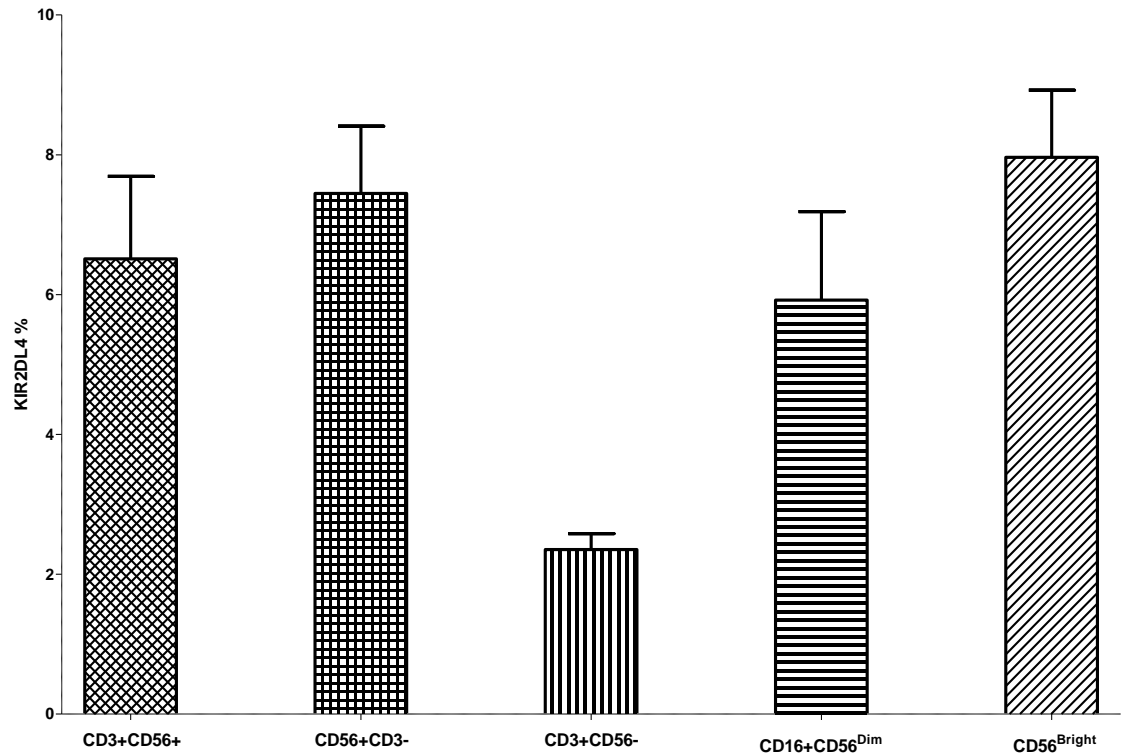


Fig. 4.4. The resting expression of KIR2DL4 on NK and non-NK cells in CMV- subjects. Results are expressed as mean \pm SEM (n = 7).

4.5. Induction of KIR2DL4 with IL-2 culture in lymphocytes from CMV- Individuals

PBMCs from CMV- subjects were cultured with IL-2, recovered and phenotyped with monoclonal Abs specific for NK cells and KIR2DL4. The data obtained are shown in Fig. 23. CD3+CD56+ cells had the highest proportion of KIR2DL4 cells (19.4%), while only 1.1% of CD56^{bright} NK cells were positive. The percentage of CD56^{dim} NK cells, CD56+CD3- cells and CD3+CD56- cells expressing KIR2DL4 were 8.5%, 6.5% and 3.4%, respectively (Fig. 4.5).

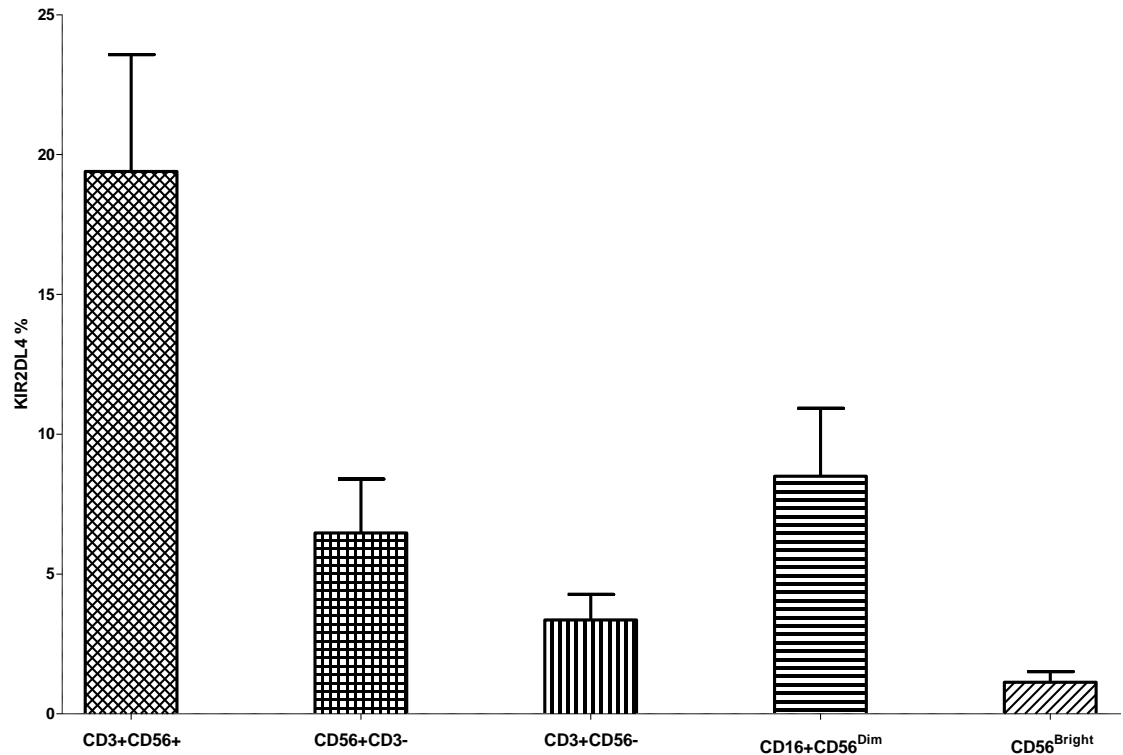


Fig. 4.5. KIR2DL4 expression on NK and non-NK cells after two weeks of IL-2 culture in CMV - participants. Results are expressed as mean \pm SEM (n = 7).

4.6. Comparison of KIR2DL4 expression on resting Lymphocytes and at D14 of IL-2 induction in CMV- individuals

Comparison of the proportions of KIR2DL4+ NK cells and other lymphocytes between resting cells and two weeks after incubation with IL-2 have shown some significant differences in CD3+CD56+ lymphocytes which upregulated the KIR2DL4 on D14 of culture ($P=0.0256$, two tailed Paired t test). Similarly, CD56^{bright} NK cells significantly downregulated KIR2DL4 after two weeks of incubation with IL-2 (0.0144). However, there were no statistically significant differences between the D0 and D14 of culture for other cell populations tested ($P>0.05$; Fig. 4.6).

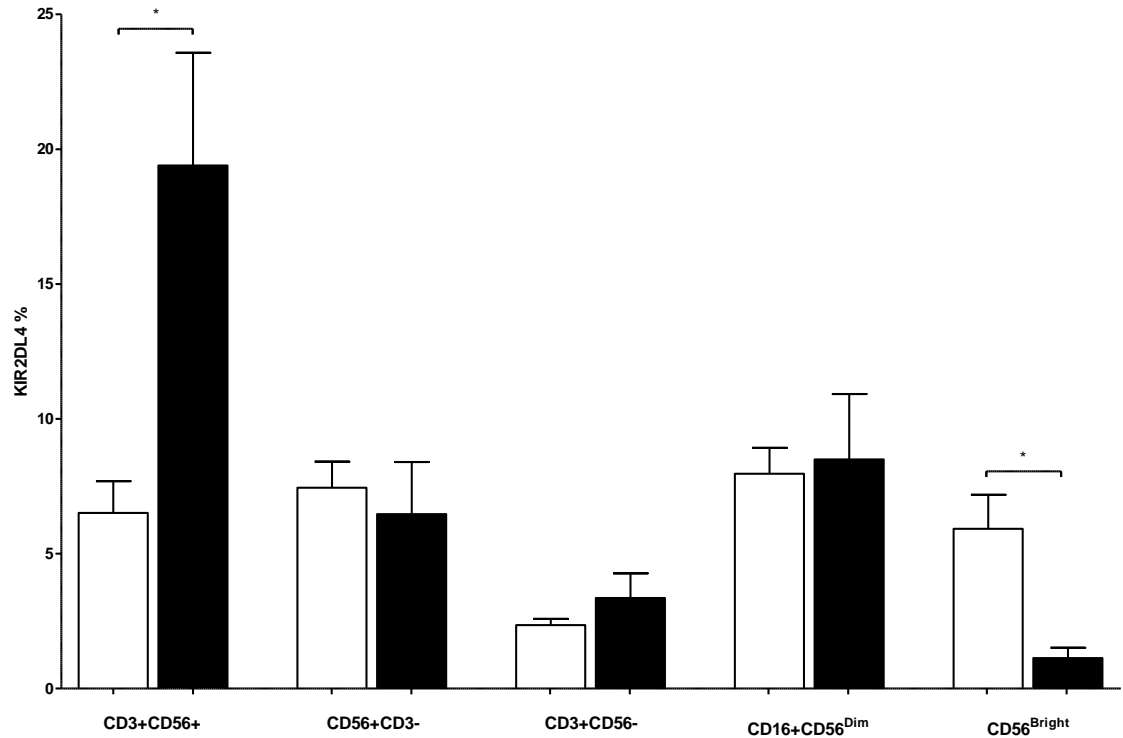


Fig. 4.6. KIR2DL4 expression on resting cells (open columns) and following two weeks incubation with IL-2 (filled columns) in CMV- subjects. Results are expressed as mean +/- SEM (n = 7), P<0.05.

4.7. KIR2DL4 expression on resting NK cells and lymphocytes in CMV+ and CMV- subjects

The proportions of NK and non-NK cells expressing KIR2DL4 in CMV+ and CMV- donors were compared on freshly isolated cells. Overall, proportions of KIR2DL4+ cells in almost all the fresh cell populations were slightly higher in CMV+ individuals than CMV- subjects apart from CD3+CD56+ lymphocytes in which the CMV- group had more KIR2DL4 expression than the CMV+ cells. From a statistical point of view, no significant differences were noted between all the groups of cells after testing the differences with a two tailed Unpaired t test $P > 0.05$, however, only CD56^{bright} cells have shown close to significant difference with $P = 0.0821$ (Fig. 4.7).

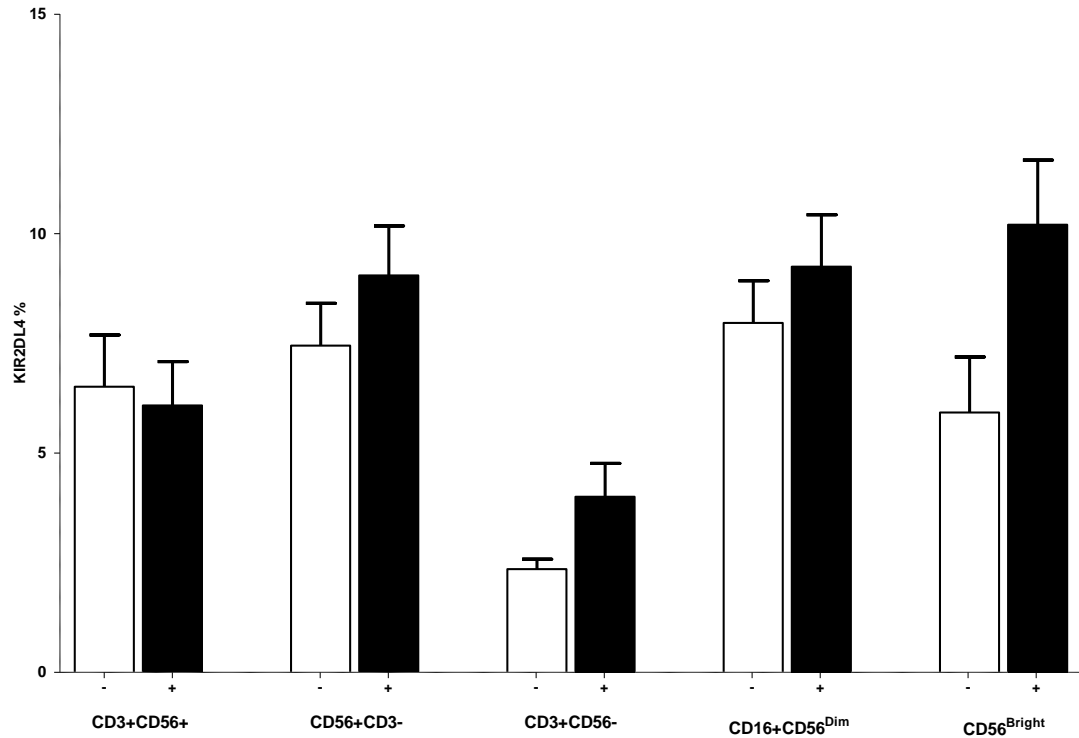


Fig. 4.7. The relative proportions of freshly isolated cells expressing KIR2DL4 in CMV- (empty columns) and CMV + (filled columns) subjects. Results are expressed as mean +/- SEM (n = 7), P>0.05.

4.8. Comparison of KIR2DL4 expression on cultured lymphocytes after IL-2 induction in CMV+ and CMV- subjects

Likewise, a comparison of the KIR2DL4 expression after culture with IL-2 in CMV + and CMV - subjects has been made (Fig. 4.8). In general, there were no statistical differences between the CMV+ and CMV- groups in all the cell populations tested when a two tailed Mann Whitney test was applied (P>0.05). However, there was a trend of higher proportions of KIR2DL4 expressing lymphocytes in CD3+CD56+, CD3+CD56- T cells and NK dim cells (CD16+CD56^{dim}) in CMV- donors than CMV+ ones while the opposite was noted in NK bright (CD56^{bright}CD16-) and the overall CD56+CD3- lymphocyte population in CMV+ subjects where higher proportions of cells expressed KIR2DL4 than in CMV- subjects.

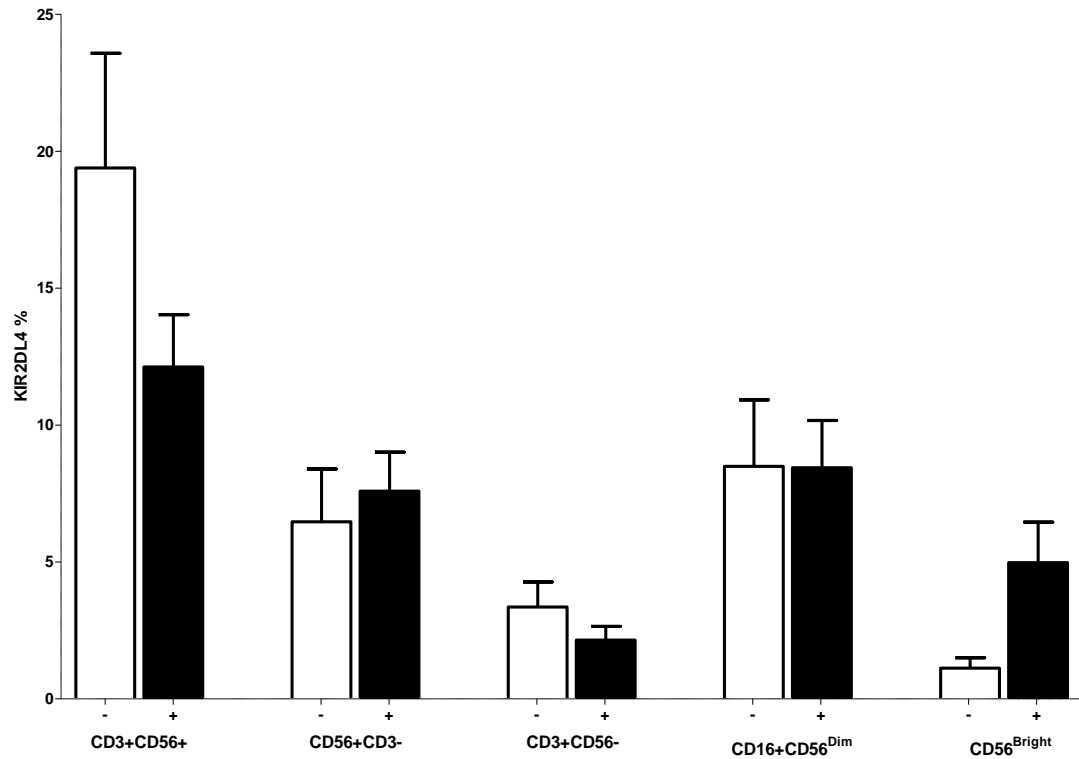


Fig. 4.8. KIR2DL4 expression on NK and non-NK cells following IL-2 culture in CMV- (empty columns) and CMV+ (filled columns) individuals. Results are expressed as mean \pm SEM (n = 7), $P > 0.05$.

4.9. KIR2DL4 Genotyping

Genotyping of the KIR2DL4 genes was carried out using a sequencing technique to identify the presence of the 9A or the 10A dimorphism in exon 6, characterized by the presence or absence of frame shifting at the last adenine and the following bases that will develop irregular intersected peaks in the sequencing pattern followed the 9A or the 10A in heterozygotes. The main point of identifying these segments was to classify those subjects as 9A or 10A who could have differing expression of KIR2DL4 upon IL-2 induction. Theoretically, the 9A genotype will not express the KIR2DL4 after two weeks of IL-2 stimulation, while those with 10A will have elevated levels of KIR2DL4 expressed after IL-2 induction for two weeks. The genotype was then analyzed in relation to KIR2DL4 expression in NK cells isolated from whole PBMCs cultured with IL-2.

According to the KIR2DL4 genotyping, the healthy donors were categorized into 9A and 10A homozygotes and heterozygotes and the expression patterns on each

group were compared in resting cells and after IL-2 culture (Fig. 4.9). In 9A homozygotes, a trend of KIR2DL4 downregulation was recorded in both CD56^{dim} and CD56^{bright} NK cells after cell culture, with significant differences in NK bright (CD56^{bright}CD16⁻) cells (P=0.0024) but not in the NK dim (CD16⁺CD56^{dim}) cells where no statistical difference was noted (P>0.05) when a Wilcoxon matched-pairs signed rank test (two tails) was applied. Unlike the CD56^{dim} and CD56^{bright} NK cells, CD3⁺CD56⁺ lymphocytes demonstrated significant increase in proportions of KIR2DL4⁺ cells after culture with IL-2 (P=0.0210). On the other hand, 10A homozygotes manifested no significant downregulation of KIR2DL4 in NK bright cells after testing the groups with a Wilcoxon matched-pairs signed rank test (two tails, P>0.05). Conversely, significantly higher proportions of CD3⁺CD56⁺ lymphocytes expressed the protein after cell culture (P=0.0029).

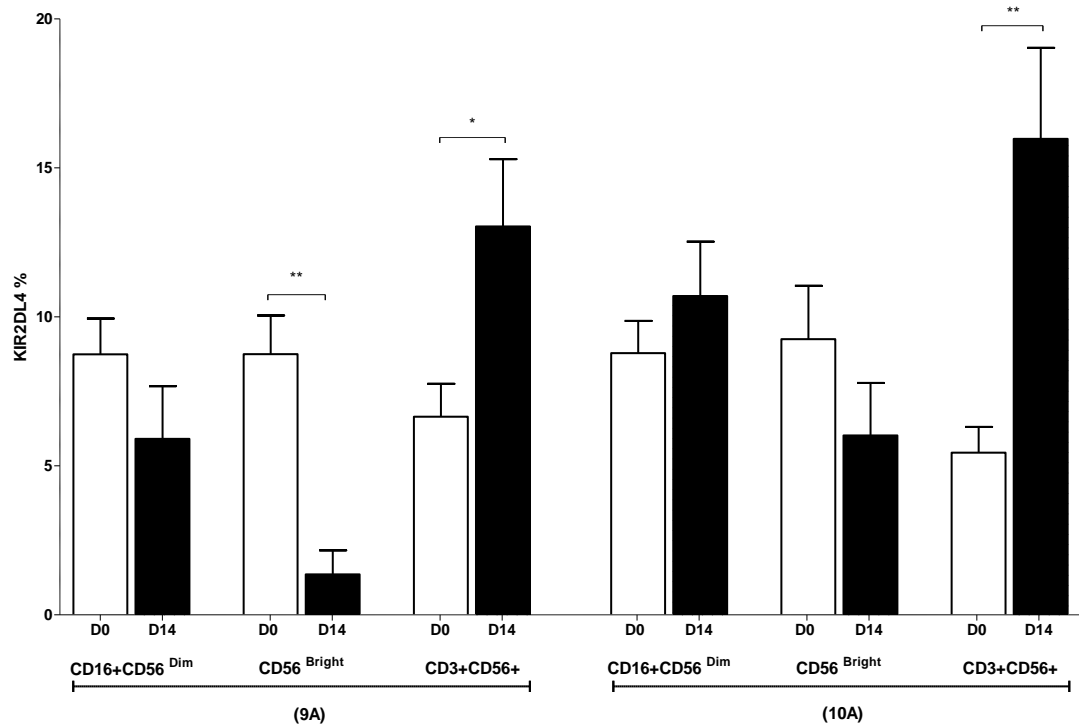


Fig. 4.9. The expression of KIR2DL4 in 9A and 10A homozygotes in resting (empty columns) and IL-2 cultured NK and non-NK cells (filled columns). Results are expressed as mean \pm SEM (n = 22), P<0.05, P<0.01.

4.10. KIR2DL4 gene expression

The relative quantitation of KIR2DL4 gene expression by NK cells isolated from whole PBMCs cultured with and without IL-2 for two weeks was evaluated by qPCR as mentioned in chapter 2 (Fig. 4.10a). Four samples were analysed using qPCR and cDNA was prepared from both stimulated and non-stimulated NK cells and run for 40 cycles. The data obtained were analysed with relative quantitation of gene expression utilising the ΔCT method that deduced the values for the house keeping genes from the tested and the control cycles, the ΔCT were then calculated with ($2^{\Delta CT}$) equation and the values were transferred to percentages. The data plotted in Fig. 4.10b indicate the difference in the KIR2DL4 gene expression by the stimulated and non-stimulated cells illustrating a significant difference between the two groups of cells ($P=0.0059$) with a two tailed Paired t test.

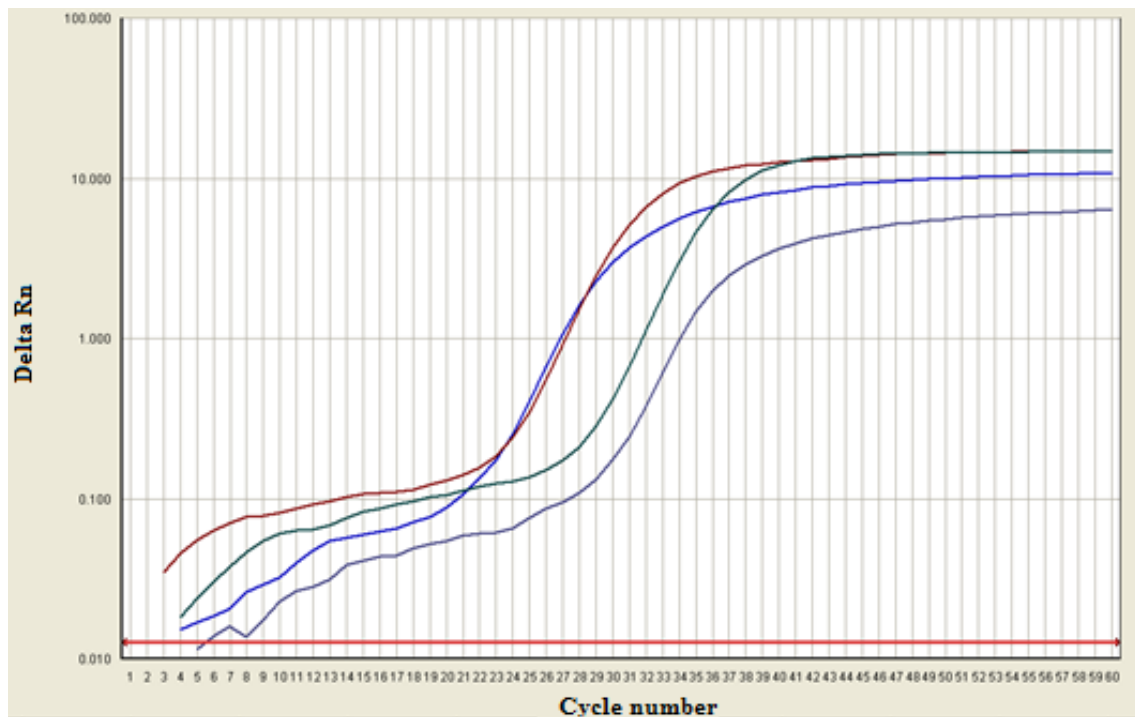


Fig. 4.10a. Relative gene expression of KIR2DL4 revealed by qPCR. The first two curves are the control (the house keeping genes), while the next two curves are the induced cells and non-induced respectively. The NK cells were isolated from whole PBMCs cultured with and without IL2.

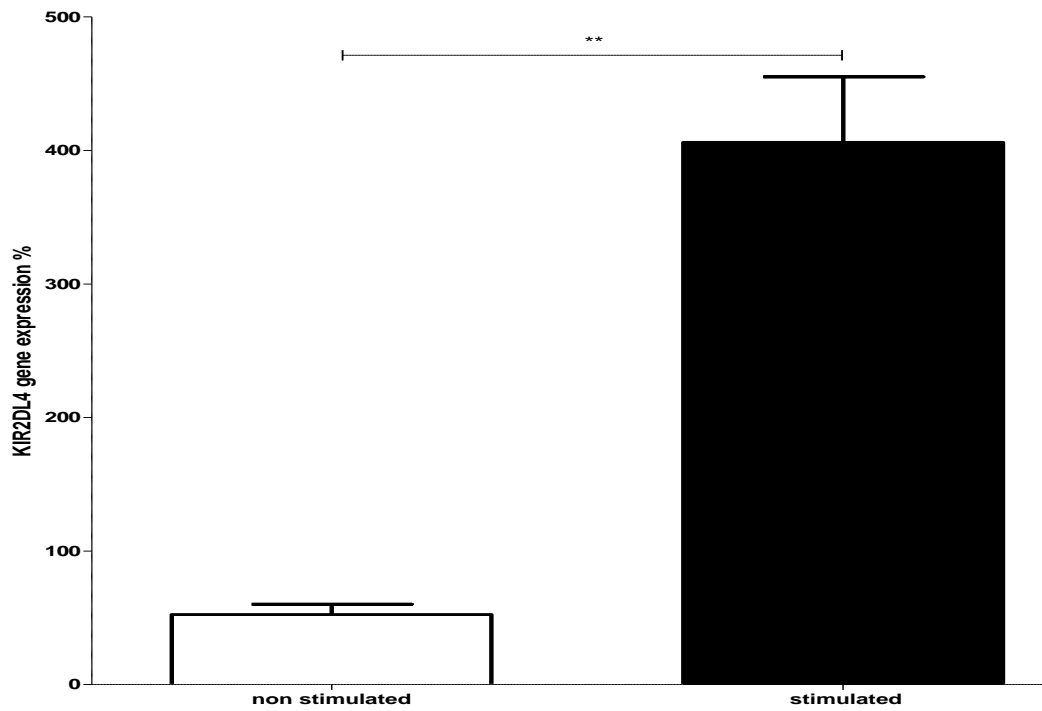


Fig. 4.10b. The gene expression of KIR2DL4 in stimulated and non-stimulated NK cells isolated from whole PBMCs culture with IL-2. Results are expressed as mean \pm SEM, $n = 4$, ** $P < 0.01$.

4.11. Summary and discussion of KIR2DL4 expression in relation to IL-2, CMV status and genotyping

To sum up, the expression of KIR2DL4 in CMV+ subject was significantly upregulated on the surface of CD3+CD56+ cell and significantly down regulated in CD56^{bright} cells. Similarly, in CMV- individuals, CD3+CD56+ lymphocytes showed significant increase of surface protein while CD56^{bright} cells was significantly downregulating the KIR2DL4 following IL-2 induction. Genotype of 9A seems to inhibit the expression of KIR2DL4 in CD56^{bright} cells and induce expression of the receptor in CD3+CD56+ lymphocytes. Whereas, the 10A allele have only seen upregulating KIR2DL4 in CD3+CD56+ lymphocytes. Real time PCR for KIR2DL4

gene expression was significantly augmented in B cells isolated from whole PBMCs stimulated with IL-2 for 2 weeks compared to control cells Table 4.1.

Figure	Molecule	Cell population	CMV
4.3	KIR2DL4	CD3+CD56+	+
4.3	KIR2DL4	CD56 ^{bright}	+
4.6	KIR2DL4	CD3+CD56+	-
4.6	KIR2DL4	CD56 ^{bright}	-
4.9	9A	CD3+CD56+	Not applicable
4.9	9A	CD56 ^{bright}	Not applicable
4.9	10A	CD3+CD56+	Not applicable
4.10	Gene expression	NK cells	Not applicable

Table 4.1. Summary of significant differences in proportions of KIR2DL4 protein and gene expression according to CMV status, 9A and 10A frequency in IL-2 stimulated cells.

The results regarding KIR2DL4 expression in healthy individuals have manifested that there was significant increment in the expression of KIR2DL4 in CD3+CD56+ lymphocytes and significant downregulation of the protein on CD56^{bright} cells following IL-2 treatment. These data are not in accordance with previous observations made by other groups who reported no cell surface expression of KIR2DL4 in naïve NK cells extracted from whole blood (J. P. Goodridge et al., 2003; Kikuchi-Maki et al., 2003). These controversial data could be due to the protein being an intracellular receptor residing in the endosomes (Rajagopalan et al., 2006) or it could be because of the mAb clone used in previous work did not recognise the cell surface receptor.

The surface expression of KIR2DL4 on cell of some resting PBMCs could suggest the possibility of this molecule being acting as act as a co-receptor that co-exists with other ligands like ILT-2 which is exhibited on NK cells, T lymphocytes and monocytes that either inhibit or activate the cells expressed on (Colonna et al.,

1997). Resting NK cells and T lymphocytes expressing KIR2DL4 could be as a part of an ongoing tolerogenic strategy exerted by this protein upon interacting with its ligand, HLA-G. This sort of interaction can take place within endosomes and may result in the modification or alteration in cytokines produced by NK cells (Rajagopalan et al., 2006; Sumati Rajagopalan & Eric O. Long, 1999). In addition, the ligation of KIR2DL4 with soluble or cell-associated HLA-G could give rise to proinflammatory cytokine production by NK cells and inhibition of cytotoxic activity of KIR2DL4 expressing cells (Kikuchi-Maki et al., 2003; Rajagopalan et al., 2006; Yu, Tian, Wang, & Feng, 2006).

On the other hand, data presented in this study demonstrated that the expression of KIR2DL4 was not influenced by the allelic polymorphism in the KIR2DL4 genes including the 9A and 10A genotypes, as the receptor was found on the cell surface in both 9A and 10A variants. This could be attributed to the presence of other genetic factors related to transcriptional or post transcriptional processes of mRNA or possibly because of the donors being involved in the study where of different ethnic background as other studies have included population from local communities and similar ethnic background.

CHAPTER FIVE

RESULTS

Effect of CMV induction on CD103 expression

CD103 is an integrin expressed by some mucosal and activated lymphocytes (Cerf-Bensussan et al., 1987; Hynes, 2002), including those infiltrating organ transplants during rejection episodes (Racusen et al., 1999). This receptor is reported to localise lymphocytes reside in epithelial tissues (Schon et al., 1999) where its ligand E-cadherin found on epithelial cells (Agace et al., 2000). This receptor was evaluated on PBMCs from healthy subjects to test whether the molecule could be upregulated on these cells which may influence their migration to epithelial tissues where CMV might enter the body and propagate. This section will cover the expression of CD103 on resting PBMCs and after stimulation with CMV antigen. A total of 10 healthy donors (50% CMV- and 50% CMV+) were recruited and a base line expression of CD103 was measured using monoclonal Abs and flow cytometry. Also, the co-expression of CD103 with HLA-G was evaluated on CD103+ PBMCs to test the possibility of CD103+HLA-G+ cells to be tolerogenic and to display regulatory function.

5.1. Expression of CD103 on resting PBMCs in CMV + donors

The expression of CD103 on various PBMCs was measured on resting cells in CMV + donors, some flow cytometry plots are shown in (Fig. 5.1a). The percentage of this receptor was highest on CD3+CD56+ cells (0.5%) and the lowest expresser was CD3-CD56+ cells (0.026%). Cytotoxic T cells ranked the second (0.398%) followed by T helper cells (0.164%) and B cells (0.07%). This receptor showed fairly low levels on resting cells with all populations displaying less than 1% CD103+ cells (Fig. 5.1b).

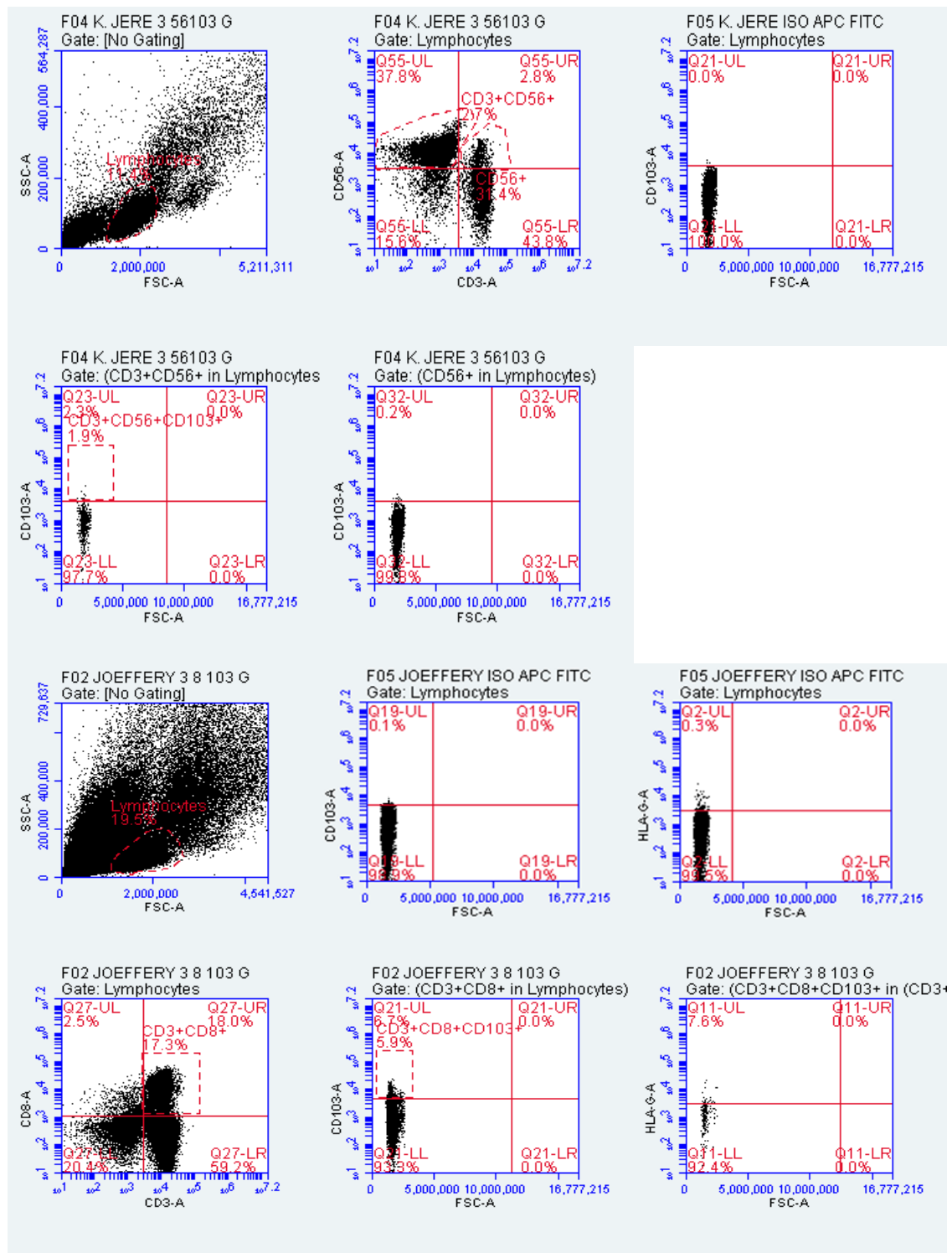


Fig. 5.1a. Acquisition plots of Accuri C6 flow cytometry exhibiting in order: scatter profile showing lymphocyte gate; CD3 and CD56 co-labelling showing gates used for CD3-CD56+, CD3+ and CD3+CD56+ cells; isotype control; CD103 proportions on CD3+CD56+ and CD3-CD56+ cells on resting day. Likewise, the lower panel of plots showing gating of CD3+CD8+ cells and further labelling with CD103 and HLA-G in the presence of isotypes.

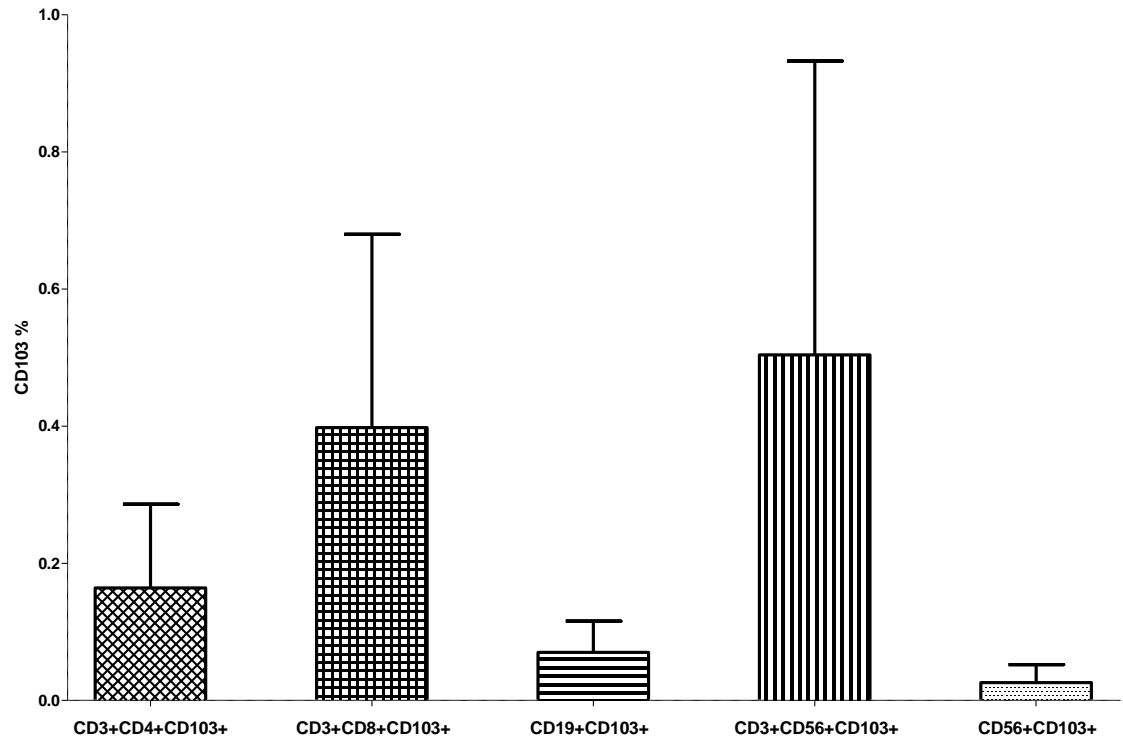


Fig. 5.1b. Expression of CD103 on various resting PBMC populations in CMV+ individuals. Results are expressed as mean \pm SEM (n = 5).

5.2. Expression of CD103 on PBMCs cultured with CMV antigen in CMV+ individuals

On day 7 of cell culture with CMV antigen, the level of CD103 was re-assessed with flow cytometry. Noticeably, the principal population which displayed the receptor was CD3+CD56+ T cells (18.8%), while, a very low level was exhibited by helper T cells (2.2%). The remaining cells displayed the molecules in the following order; B cells (8.16%), NK cells (5.8%) and cytotoxic T cells (4.56%). Interestingly, most of the PBMC subsets revealed upregulation patterns after CMV induction (Fig. 5.2).

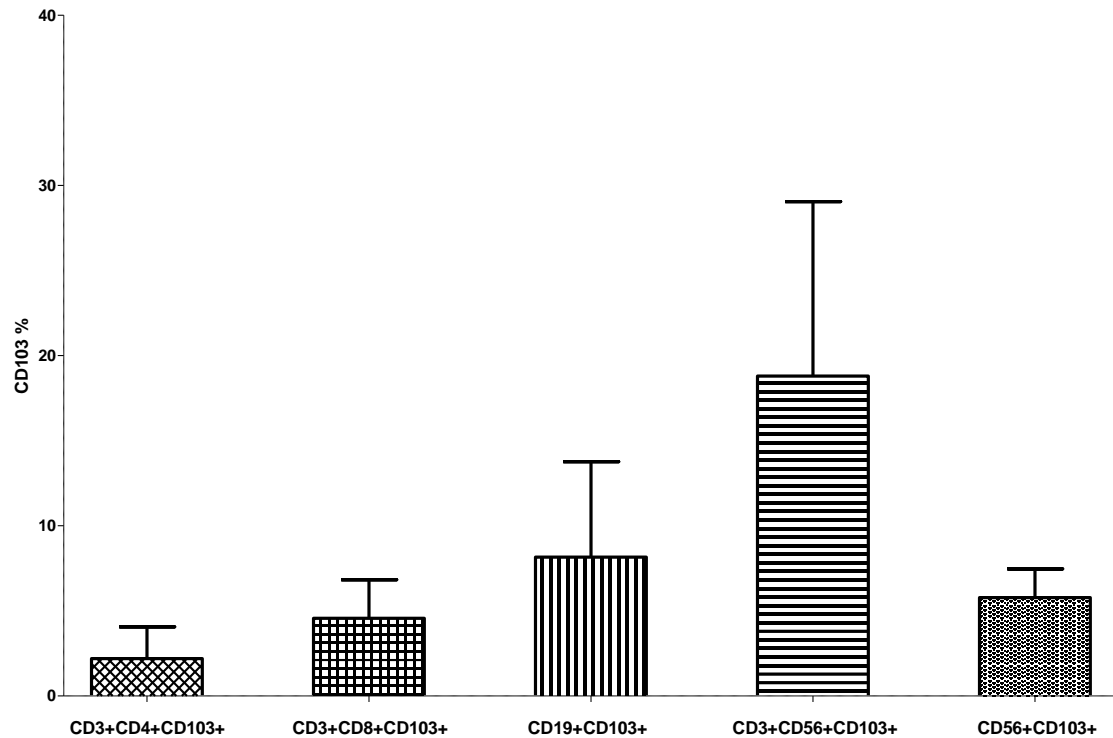


Fig. 5.2. Expression of CD103 on various PBMC populations after culture with CMV antigen in CMV+ subjects. Results are expressed as mean \pm SEM (n = 5).

5.3. CD103 expression on resting cells and after CMV induction in CMV+ donors

The proportions of CD103+ fresh cells and after CMV induction was compared to illustrate the induction levels after cell stimulation (Fig. 5.3). Apparently, there was clear upregulation of the receptor on all the groups of cells tested. However, when a Wilcoxon matched-pairs signed rank test (two-tailed) was performed to detect the difference level between resting and after cell induction, CD3+CD56+ T cells exhibited significantly increased percentage of CD103+ cells ($P=0.0313$). Similarly, NK cells have also showed a significant increase in the % positive cells following cell stimulation with viral antigen ($P=0.0313$). All the remaining group of cells including T helper cells, cytotoxic T cells and B cells did not show significant changes ($P>0.05$). However, for B cells the increase was not quite statistically significant ($P=0.0625$).

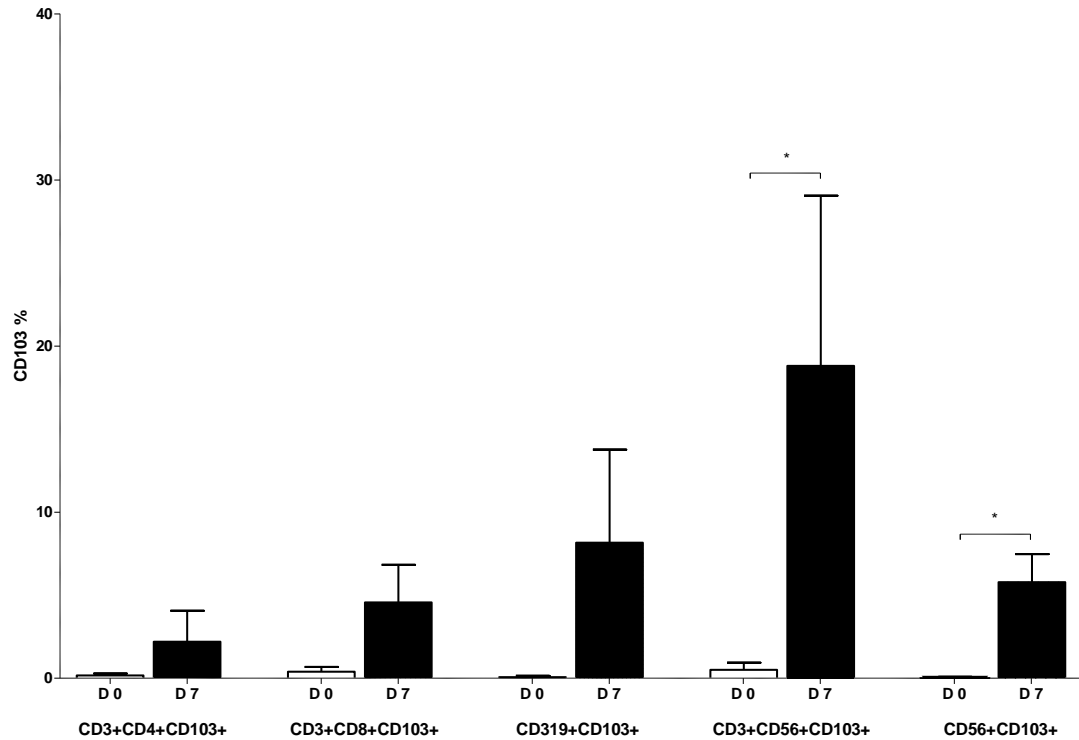


Fig. 5.3. CD103 comparison on various PBMC populations on resting cells (empty columns) and following culture with CMV antigen (filled columns) in CMV + subjects. Results are expressed as mean \pm SEM (n = 5). * p<0.05.

5.4. Expression of CD103 on resting PBMCs from CMV- donors

Likewise, the expression level of CD103 was assessed on PBMCs freshly isolated from CMV- subjects (Fig. 5.4). The highest expressing subset was CD3+CD56+ cells (1.512%) and the least expressing ones were NK cells (0.066%). The other cell subsets exhibited very low percentages of the protein ranging between 0.162% for B cells, 0.148% for cytotoxic T cells and 0.092% for T helper cells.

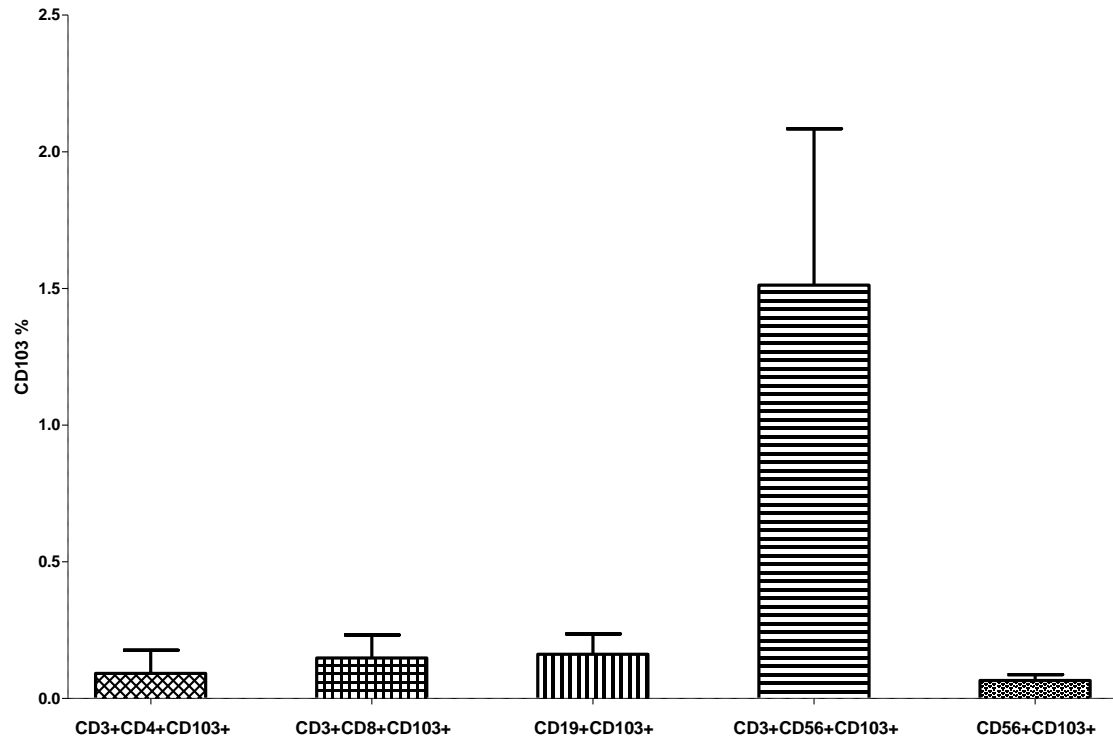


Fig. 5.4. CD103 expression on various PBMC populations on resting in CMV - subjects. Results are expressed as mean \pm SEM (n = 5).

5.5. Expression of CD103 on PBMCs cultured with CMV Antigen in CMV- subjects

On D7 of stimulation with CMV antigen, the proportion of CD103 expressing cells was measured (Fig. 5.5). The graph revealed an upregulation trend of the receptor on almost all the cell subsets tested with the highest proportion on CD3+CD56+ T cells (8.84%) and only a tiny amount was recorded on T helper cells (0.084%) while B cells, Cytotoxic T cells and NK cells expressed 4.872%, 2.764% and 2.754% respectively.

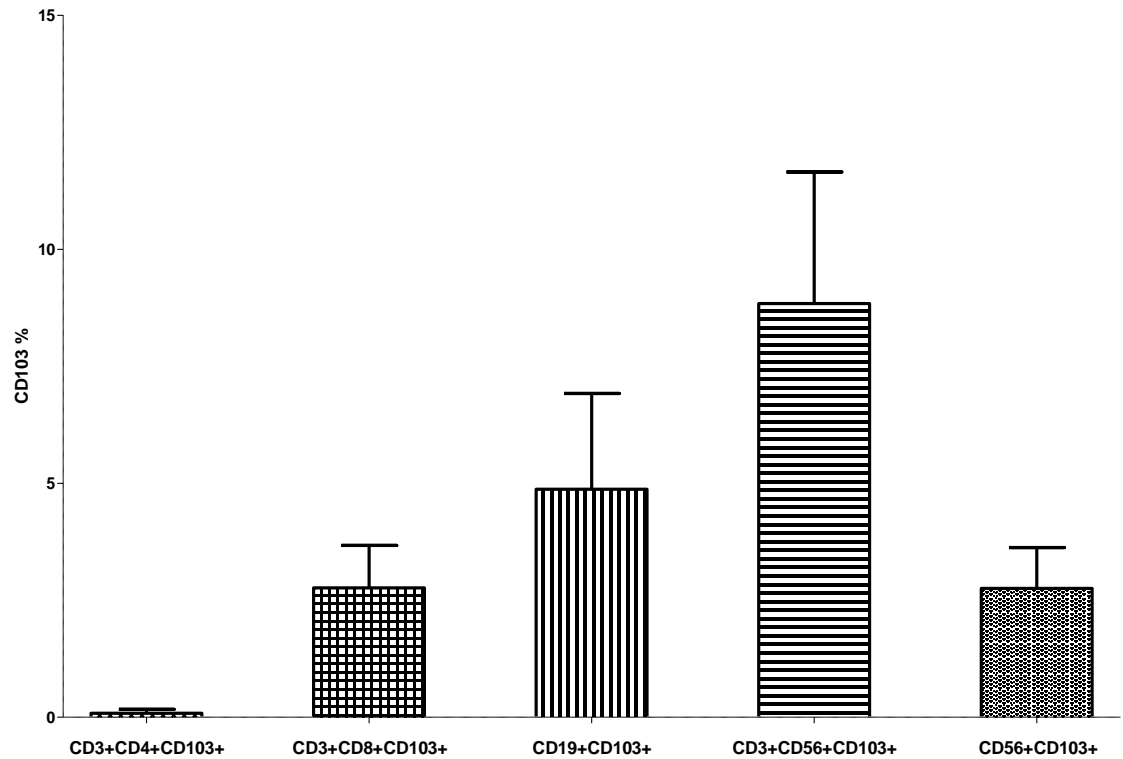


Fig. 5.5. CD103 expression on various PBMC populations after culture with CMV antigens in CMV - subjects. Results are expressed as mean \pm SEM (n = 5).

5.6. CD103 comparison on fresh cells and after 7 days of culture with CMV antigen in CMV- subjects

Following on from the two graphs above in CMV- donors, a comparison of CD103 expression before and after culture with CMV antigens was made (Fig. 5.6). Proportions of all cell subsets, with the exception of CD4+ T cells, were elevated to significant levels ($P < 0.05$) upon testing the data with a two tailed Paired t test (CD3+CD8+CD103 $P = 0.0252$, CD19+CD103+ $P = 0.0409$, CD3+CD56+C103+ $P = 0.0421$, CD56+CD103+ $P = 0.0181$).

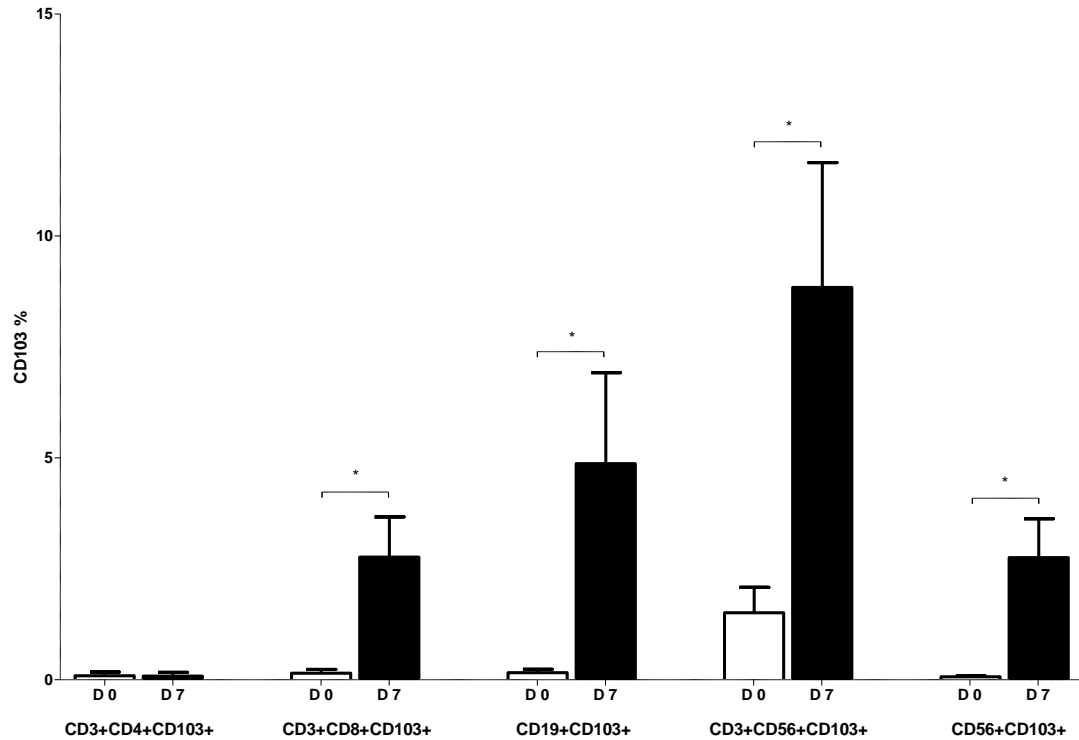


Fig. 5.6. Comparison of CD103 expression on various PBMC populations on resting cells (empty columns) and after culture (filled columns) in CMV- subjects. Results are expressed as mean \pm SEM (n = 5), * P<0.05.

5.7. Comparison of CD103 expression on freshly isolated cells in CMV- and CMV+ donors

Since the tested groups involved in the assessment of CD103 were CMV- and CMV+, a comparison was made between the two groups to detect any differences in the receptor levels expressed by various PBMCs. When a two tailed Mann Whitney test was performed there were no statistically significant differences between all the assessed cell populations ($P>0.05$) despite the apparent variations in groups, particularly in CD3+CD56+ T cells and cytotoxic T cells (Fig. 5.7).

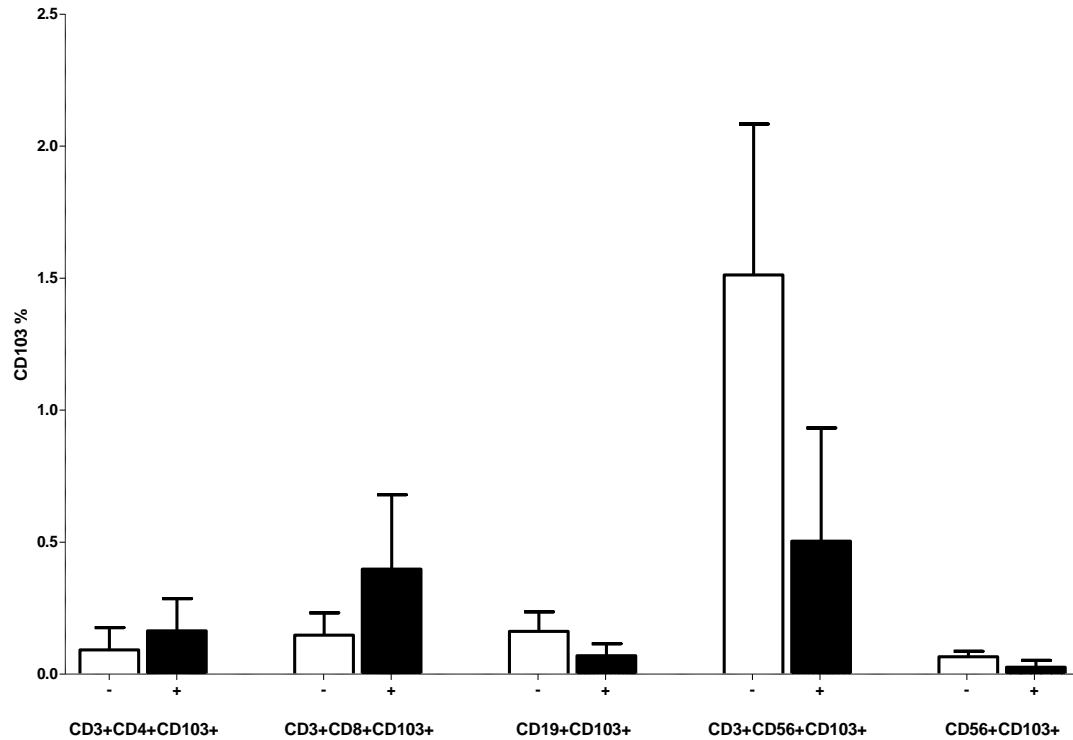


Fig. 5.7. Comparison of CD103 expression on freshly isolated PBMC populations in CMV - (empty columns) and CMV + (filled columns) subjects. Results are expressed as mean \pm SEM (n = 5 in each group). No statistically significant differences were found between groups.

5.8. Comparison of CD103 expression after stimulation with CMV antigen in CMV- and CMV+ individuals

In the same way, to detect whether there were statistical differences between CMV- and CMV+ groups in the level of CD103 expression on various PBMCs after cell culture with CMV antigen, a two tailed Mann Whitney test was applied and the P value was not significant ($P > 0.05$) in all cell populations regardless the manifested variations between the CMV- and CMV+ groups which exhibited more CD103 being expressed on the latter (Fig. 5.8).

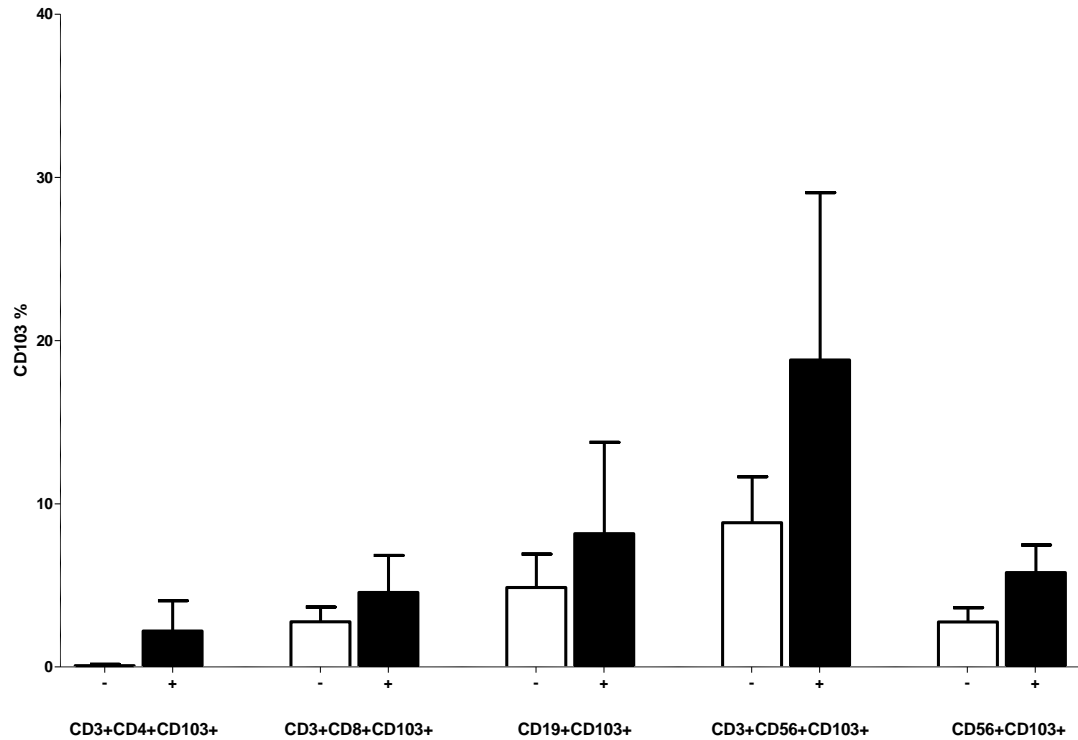


Fig. 5.8. Comparison of CD103 expression on PBMC populations after culture in CMV- (empty columns) and CMV+ (filled columns) subjects. Results are expressed as mean \pm SEM (n = 5 in each group). No statistically significant differences were found between groups.

5.9. HLA-G co-expression with CD103 on resting PBMCs in CMV + donors

The association of HLA-G with CD103 expressing cells was evaluated in the same donors on freshly isolated cells (Fig. 5.9). In CMV+ subjects, the proportion of HLA-G+CD103+ cells were greatest in CD3+CD56+ T cells (2.096%) and the least (0.272%) was in T helper cells. However, low proportions of HLA-G+ cells expressing CD103+ cells were shown by B cells (0.862%), NK cells (0.296%) and cytotoxic T cells (0.274%).

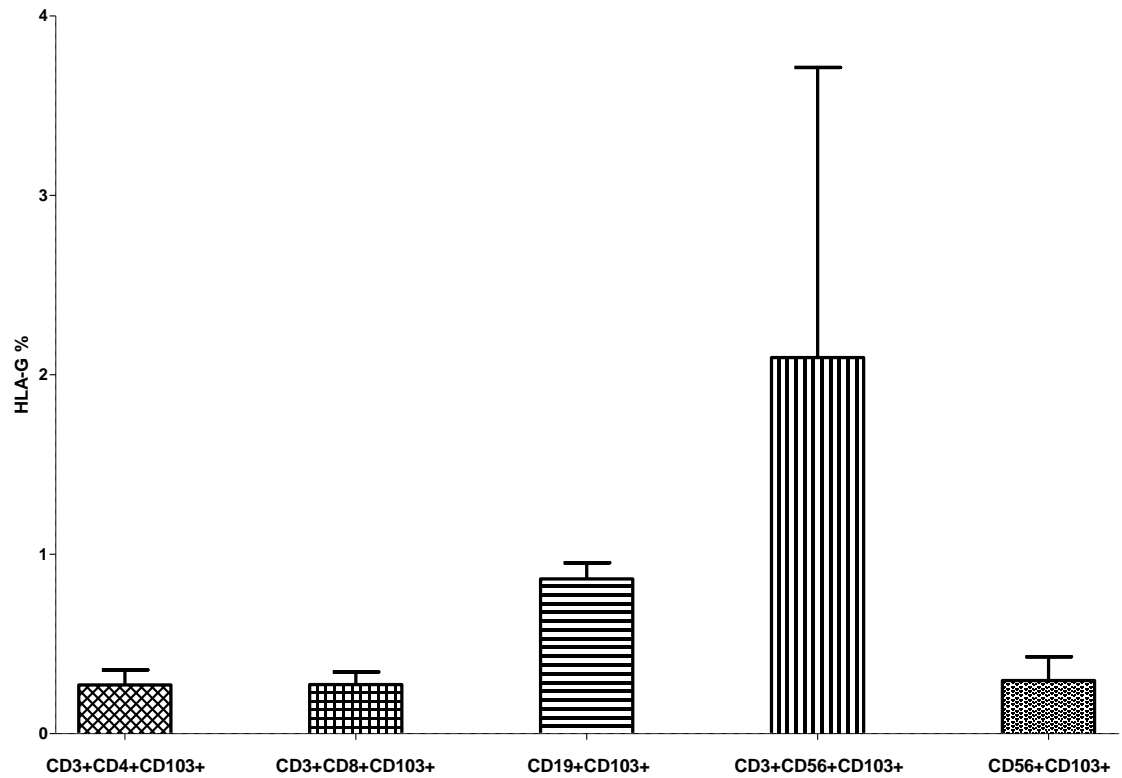


Fig. 5.9. HLA-G+CD103+ expression on various freshly isolated PBMC subsets in CMV+ donors. Results are expressed as mean \pm SEM (n = 5).

5.10. HLA-G expression in CD103+ PBMCs after culture with CMV antigen in CMV+ subjects

The expression of HLA-G on CD103+ cells after culture with CMV antigen for a week in CMV sero positive donors was evaluated and there was marked elevation of the proportions of cells expressing HLA-G (Fig. 5.10). B cells were the main expresser of HLA-G (24.03%) and the lowest proportion was found in NK cells (11.58%). The other lymphocytes co-expressed HLA-G and CD103 in the following descending order; T helper cells (20.85%), cytotoxic T cells (17.72%) and CD3+CD56+ T cells (11.76%).

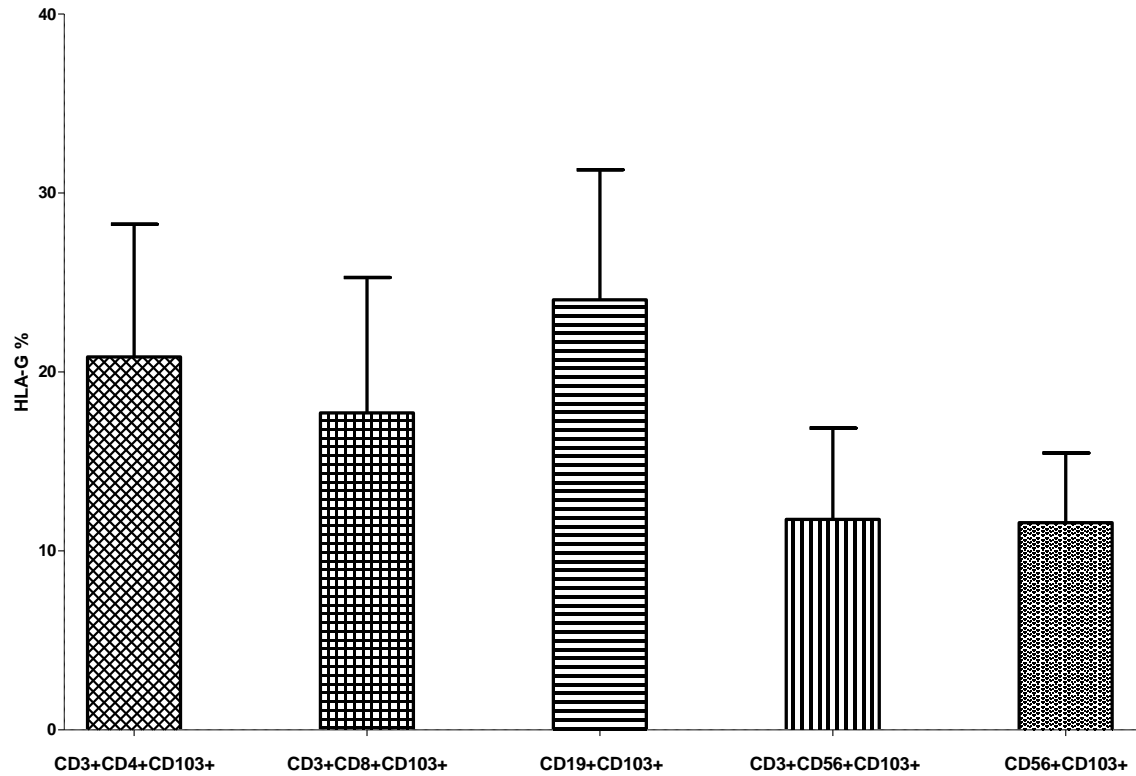


Fig. 5.10. HLA-G and CD103 co-expression on PBMC populations following culture with CMV antigen in CMV+ donors. Results are expressed as mean \pm SEM (n = 5).

5.11. Comparison of HLA-G expression on CD103+ PBMCs on fresh cells and after culture with CMV antigens in CMV + donors

The results from fresh cells and after CMV culture were then compared in CMV+ subjects to identify the differences following CMV stimulation (Fig. 5.11). To compare the difference between D0 and D7, a two tail Paired t test was applied on all the tested cell populations and most of the groups displayed significantly higher proportions of HLA-G on CD103+ cells after culture with CMV antigen with (P= 0.0252) for T helper cells, (P=0.0407) for cytotoxic T cells, (P= 0.0169) for B cells and P= 0.0208 for NK cells. Unlike, CD3+CD56+ T cells which revealed no statistical difference (P=0.1563) after cell induction with CMV antigen.

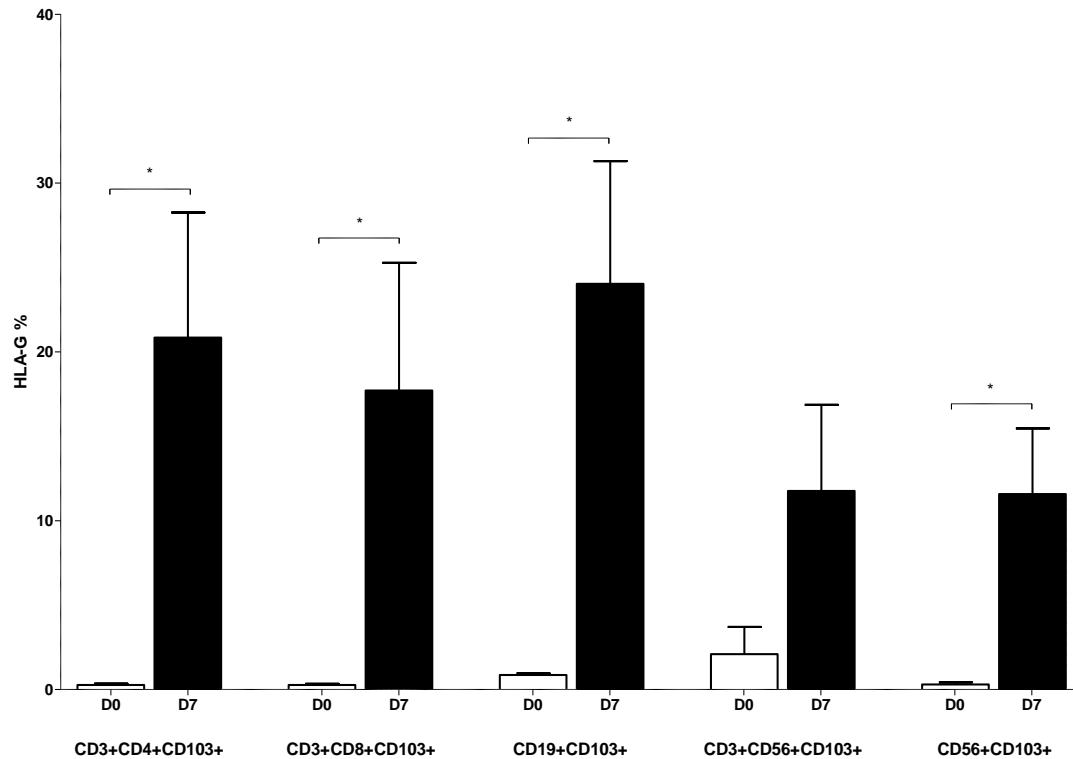


Fig. 5.11. Comparison of HLA-G+CD103+ cell proportions on fresh cells (open columns) and following culture with CMV antigen (filled columns) in CMV+ donors. Results are expressed as mean +/- SEM (n = 5 for each group). * P<0.05.

5.12. HLA-G co-expression with CD103+ in resting PBMCs in CMV- individuals

In CMV- subjects, HLA-G expressed on CD103 cells was measured on freshly isolated PBMCs using flow cytometry. Most of the tested lymphocyte subsets showed relatively low proportions of HLA-G+ cells in the CD103+ population with CD3+CD56+ being the principal lymphocytes that were positive (2.358%), whereas cytotoxic T cells showed the least proportion of the receptor (0.23%). The proportion of B cells expressing CD103 and HLA-G was (0.666%), on NK cells (0.39%) and on T helper cells (0.268%) (Fig. 5.12).

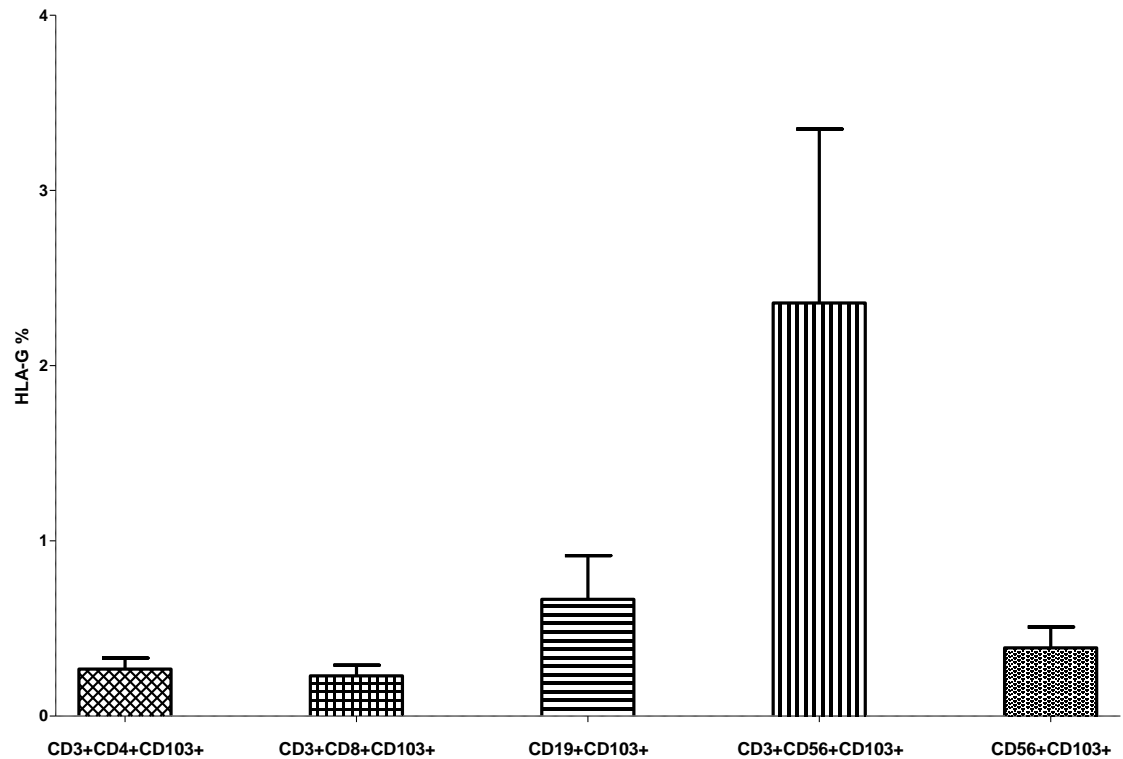


Fig. 5.12. HLA-G expression on CD103+ cells in freshly isolated PBMCs from CMV-donors. Results are expressed as mean \pm SEM (n = 5).

5.13. The co-expression of HLA-G with CD103 after culture with CMV antigen in CMV- subjects

On day 7 of cell culture with CMV antigen, there was a distinct increase of HLA-G+CD103+ expressing cells in virtually all the lymphocyte subpopulations (Fig. 5.13). The dominant lymphocytes expressing HLA-G were B cells (17.266%) and CD3+CD56+ T cells (17.006%), slightly below, NK cells (16.942%) and T helper cells (16.692%) ranked the second and finally cytotoxic T cells (12.05%) were the least.

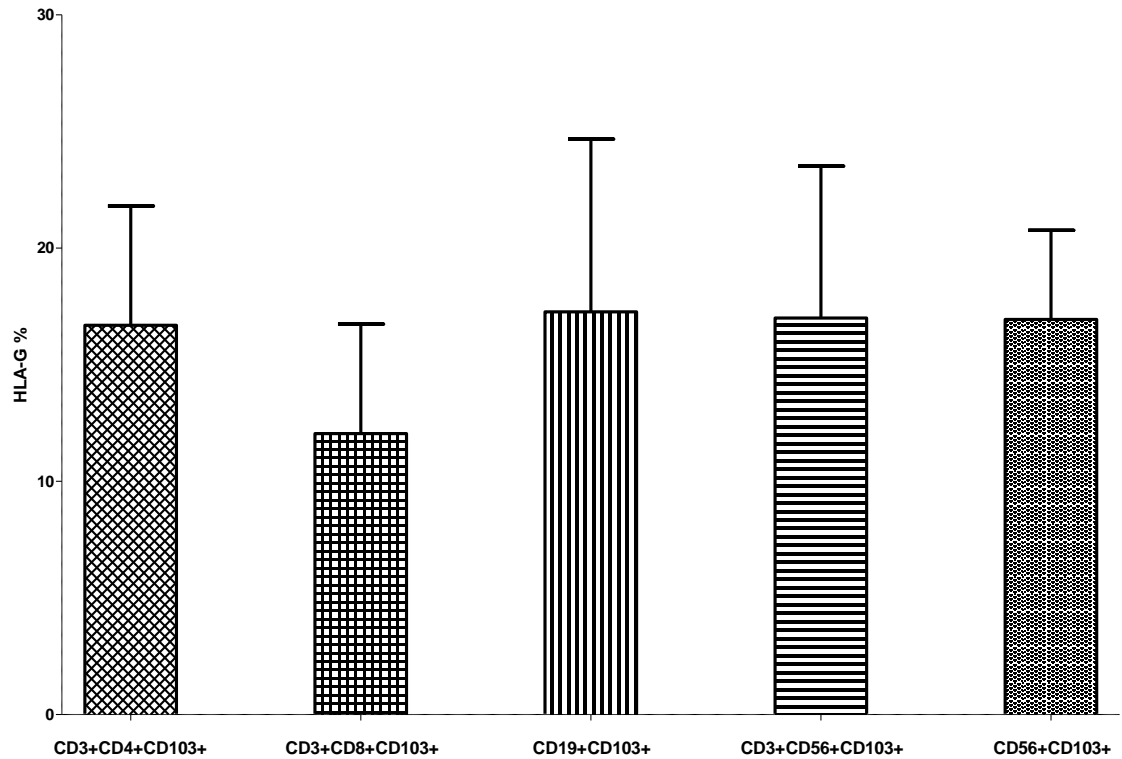


Fig. 5.13. HLA-G+CD103+ expression on PBMC populations after culture with CMV antigen in CMV⁻ donors. Results are expressed as mean \pm SEM (n = 5).

5.14. Comparison of HLA-G expression on CD103+ on freshly isolated PBMCs and following culture in CMV⁻ donors

In order to elucidate the effect of CMV antigen on the level of HLA-G on CD103⁺ cells in the CMV⁻ group, differences between all groups were tested with two tailed Paired t test (Fig. 5.14). Importantly, T helper cells expressing CD103 receptor demonstrated significant upregulation of HLA-G following viral induction ($P=0.0157$). Equally, cytotoxic T⁺ cells CD103⁺ showed significant upregulation of HLA-G after culture ($P=0.0332$). Likewise, in B cells CD103⁺ on D7 there was significant increase of HLA-G expression with $P=0.0447$. Correspondingly, NK cells CD56+CD103⁺ produced significant amount of HLA-G on D7 in contrast to D0 ($P=0.0061$). Nevertheless, from a statistical point of view, CD3+CD56+CD103⁺ T cells did not reveal significant difference on D7 ($P=0.0550$) despite the prominent upregulation of HLA-G on D7 and the P value was just below significance.

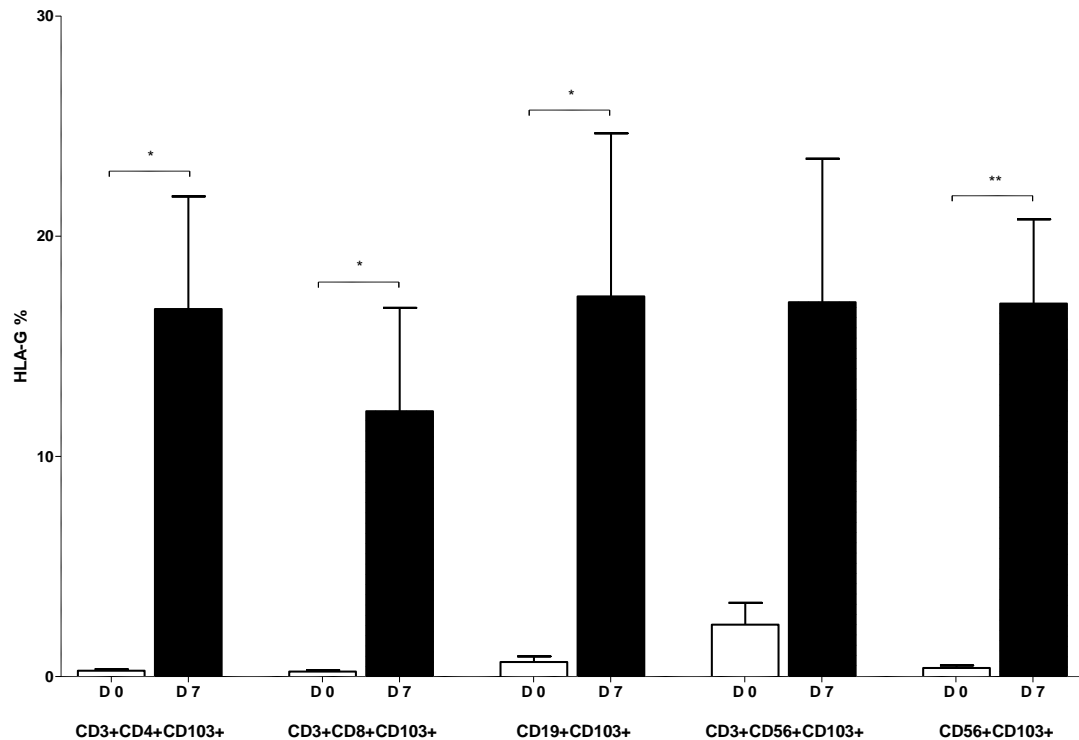


Fig. 5.14. Comparison of % of freshly isolated HLA-G+CD103+ PBMCs (empty columns) and following culture (filled columns) in CMV- donors. Results are expressed as mean \pm SEM (n = 5 in each group). * P<0.05 and **P<0.01.

5.15. Comparison of HLA-G expression on CD103+ cells in freshly isolated cells from CMV- and CMV + donors

Regarding HLA-G expression on CD103+ cells in both CMV- and CMV+ subjects, data from freshly isolated PBMCs were plotted in a comparative graph (Fig. 5.15). Consequently, when a two tailed Unpaired t test was used to detect the difference between CMV- and CMV+ subjects, no statistical differences were detected in the subpopulations of lymphocytes with $P>0.05$.

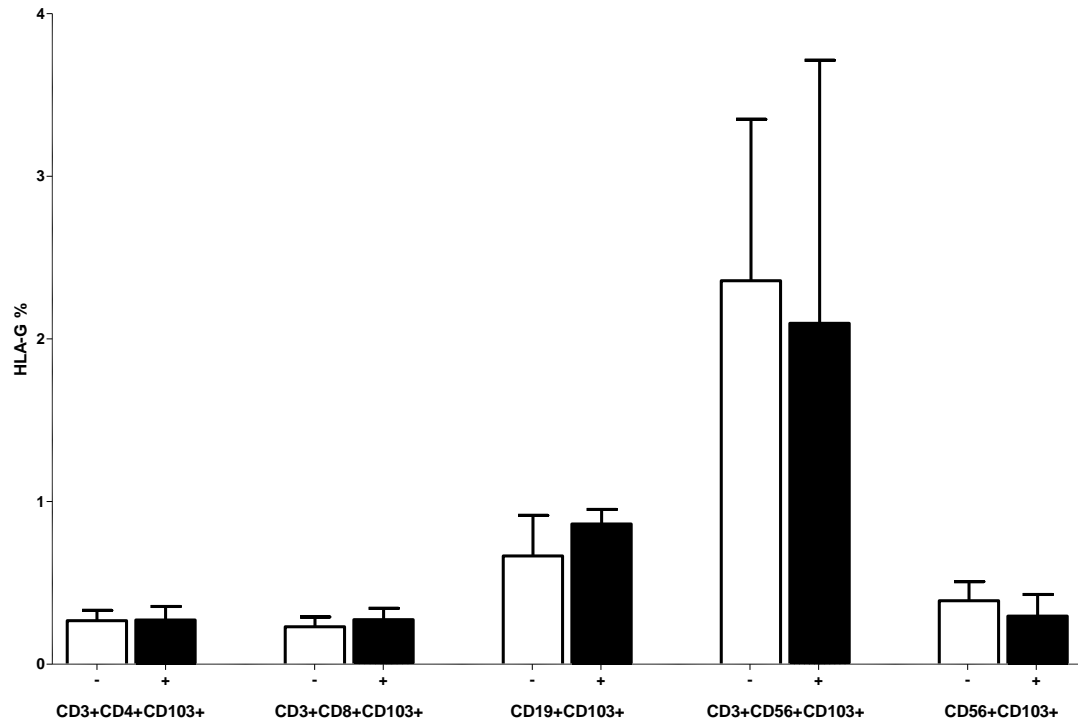


Fig. 5.15. Comparison of HLA-G expressed by freshly isolated CD103+ PBMC populations in CMV- (empty columns) and CMV+ (filled columns) subjects. Results are expressed as mean \pm SEM (n = 5 for each group). No statistically significant differences were seen between CMV+ and CMV- groups.

5.16. Comparison of HLA-G expression on CD103+ PBMCs following culture in CMV- and CMV+ groups

As far as expression of HLA-G in CD103+ lymphocytes are concerned, data from D7 of CMV antigen stimulation were combined in (Fig. 5.16) and to test for any differences in HLA-G expression among CMV - and CMV + subjects a two tailed Mann Whitney test was used, the test revealed no significant differences between all the examined cells with $P > 0.05$ for all compared populations.

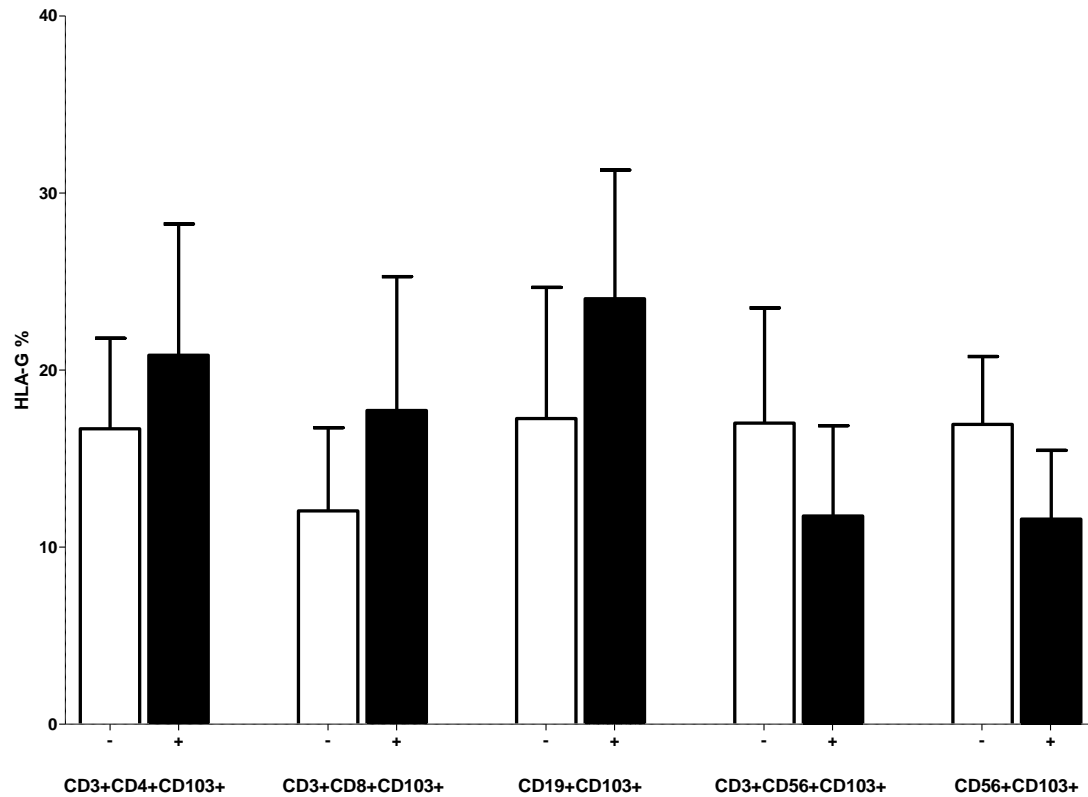


Fig. 5.16. Comparison of % of HLA-G+CD103+ PBMC populations following culture in CMV- (empty columns) and CMV+ (filled columns) individuals. Results are expressed as mean \pm SEM (n = 5 for each group). No statistically significant differences were seen between CMV+ and CMV- groups.

5.17. Expression of CD103 by resting PBMCs

Data were reanalysed by combining both CMV+ and CMV- subjects (n=10). The expression of CD103 was assessed on various freshly isolated PBMCs from CMV+ and CMV- subjects (Fig. 5.17) and the proportion of this receptor was quite low on most of the tested subsets with highest (1%) being expressed by CD3+CD56+ lymphocytes. The remaining cells showed the following proportions (CD3+CD8+= 0.27%, CD3+CD4+= 0.12%, CD19+=0.11 and CD56+=0.04%)

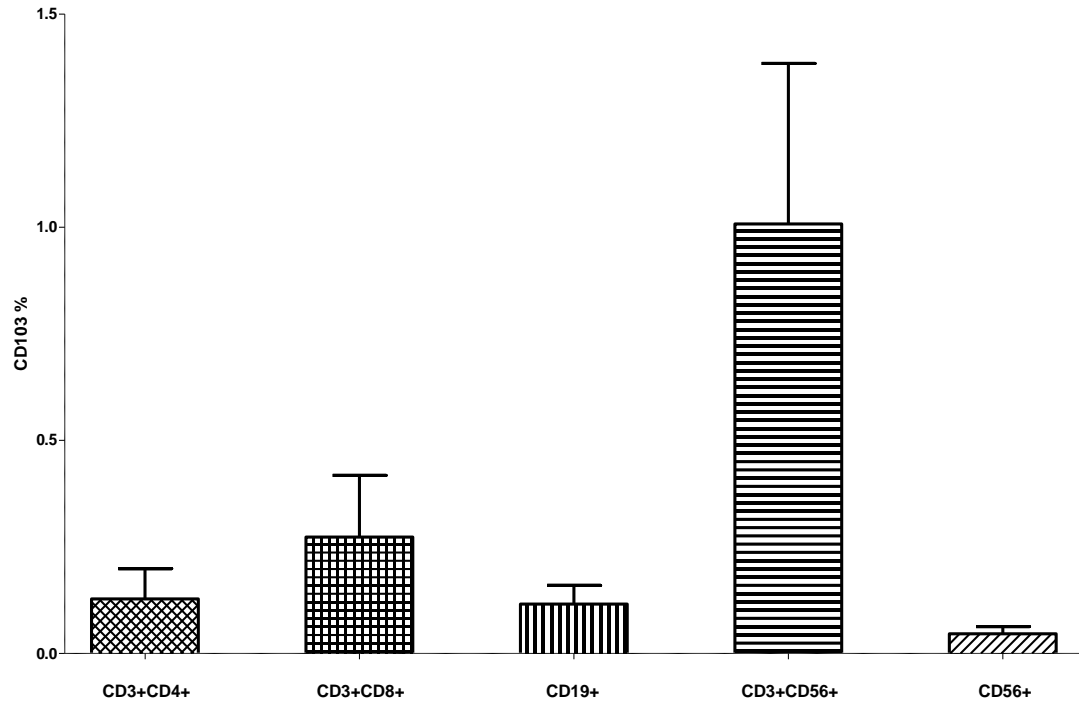


Fig. 5.17. CD103 expression on various freshly isolated PBMC from CMV + and CMV – healthy subjects. Results are expressed as mean \pm SEM (n = 10).

5.18. Expression of CD103 by PBMCs cultured with CMV antigen

Following culture with CMV antigen for a week, CD103 expression was evaluated in response to CMV induction (Fig. 5.18) and there was overall upregulation of this protein in most of the tested PBMC sets, especially CD3+CD56+ (13.82%). The other subpopulations showed the following proportions; CD19+=6.51%, CD56+=4.27%, CD3+CD8+=3.66% and CD3+CD4+=1.14%.

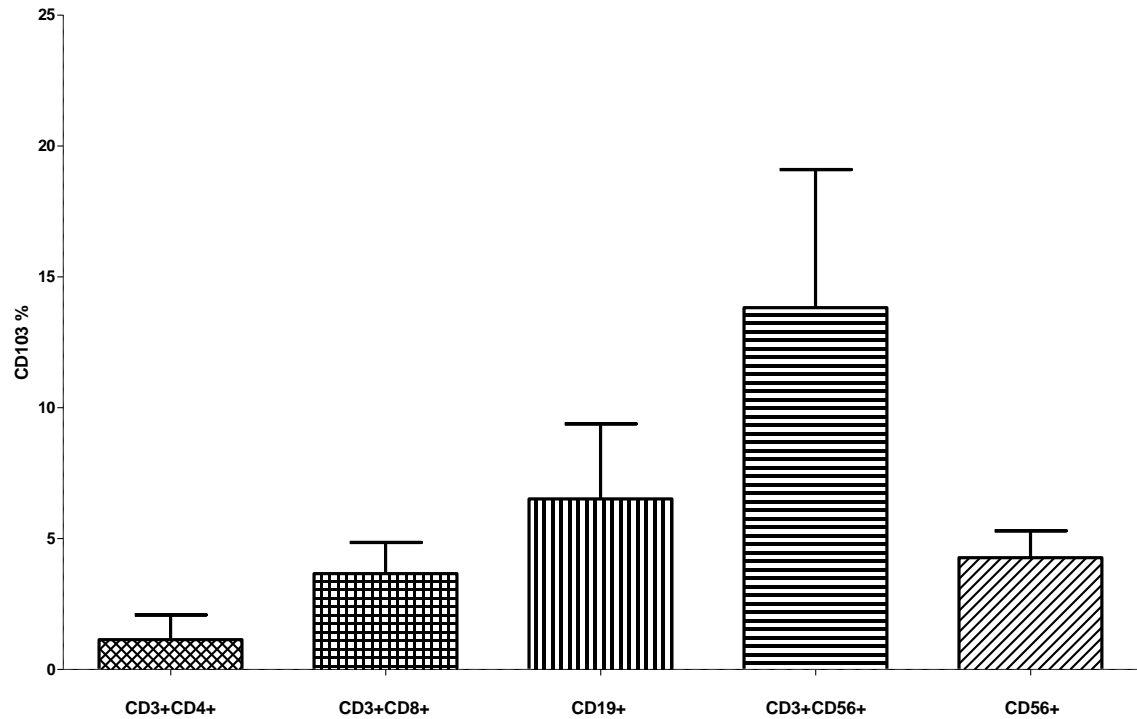


Fig. 5.18. CD103 expression in PBMCs cultured with CMV antigens for a week in CMV- and CMV+ volunteers. Results are expressed as mean \pm SEM (n = 10).

5.19. Comparison of CD103 expression on fresh cells and after CMV culture

The proportions of CD103 expressed by PBMCs at resting day and after CMV induction were plotted below (Fig. 5.19) and to test the differences between PBMC groups using a Paired t test (two tails), significant differences were recorded among CD3+CD8+ cells ($P = 0.0128$), CD3+CD56+ lymphocytes ($P = 0.0364$) and CD56+ cells ($P = 0.0028$), while, no significant differences were obtained between CD3+CD4+ ($P = 0.3093$) and CD19+ groups ($P = 0.0518$).

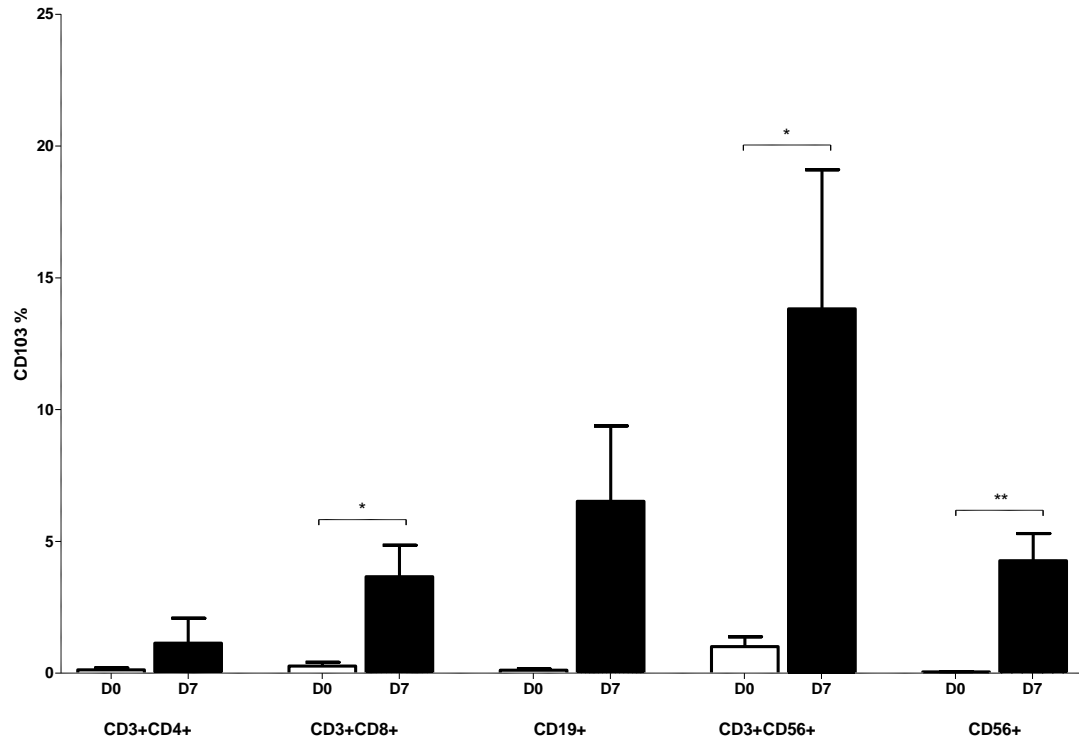


Fig. 5.19. Comparison of CD103 expressed by freshly isolated PBMC populations in CMV - (empty columns) and CMV induced cells (filled columns). Results are expressed as mean \pm SEM (n = 10). *P<0.05, **P<0.005.

5.20. HLA-G expression by CD103+ PBMCs at resting day

In parallel with CD103 assessment, HLA-G expression levels were also measured on CD103+ PBMCs (Fig. 5.20) at rest in mixed CMV+ and CMV- subjects. Those CD103+ PBMCs have shown low proportions of HLA-G+ cells with the highest (2.22%) being displayed by CD3+CD56 cells, whereas, the remaining subpopulations showed low proportions of less than 1% (CD19+= 0.76%, CD56+= 0.34%, CD3+CD4+= 0.27% and CD3+CD8+= 0.25%).

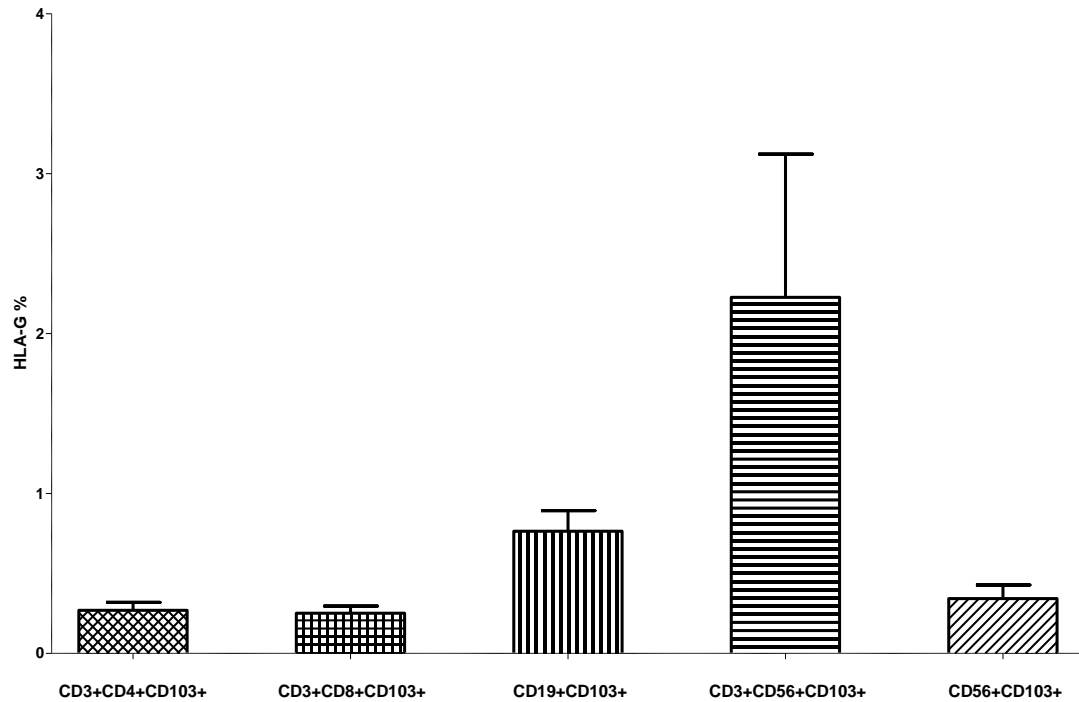


Fig. 5.20. HLA-G expressed by freshly isolated CD103+ PBMC populations. Results are expressed as mean \pm SEM (n = 10).

5.21. HLA-G expression by CD103+ PBMCs following CMV culture

Similarly, after CMV induction for a week, the proportions of HLA-G+ cells among CD103+ PBMC were measured with Accuri C6 flow cytometry and as depicted in (Fig. 5.21) all the induced PBMC sets showed increase proportions of HLA-G+ CD103+ cells with the highest (20.64%) on CD19+ cells while similar percentages were expressed by the remaining cells (CD3+CD4+= 18.78%, CD3+CD8+= 14.88 %, CD3+CD56+= 14.38 % and CD56+= 14.26%).

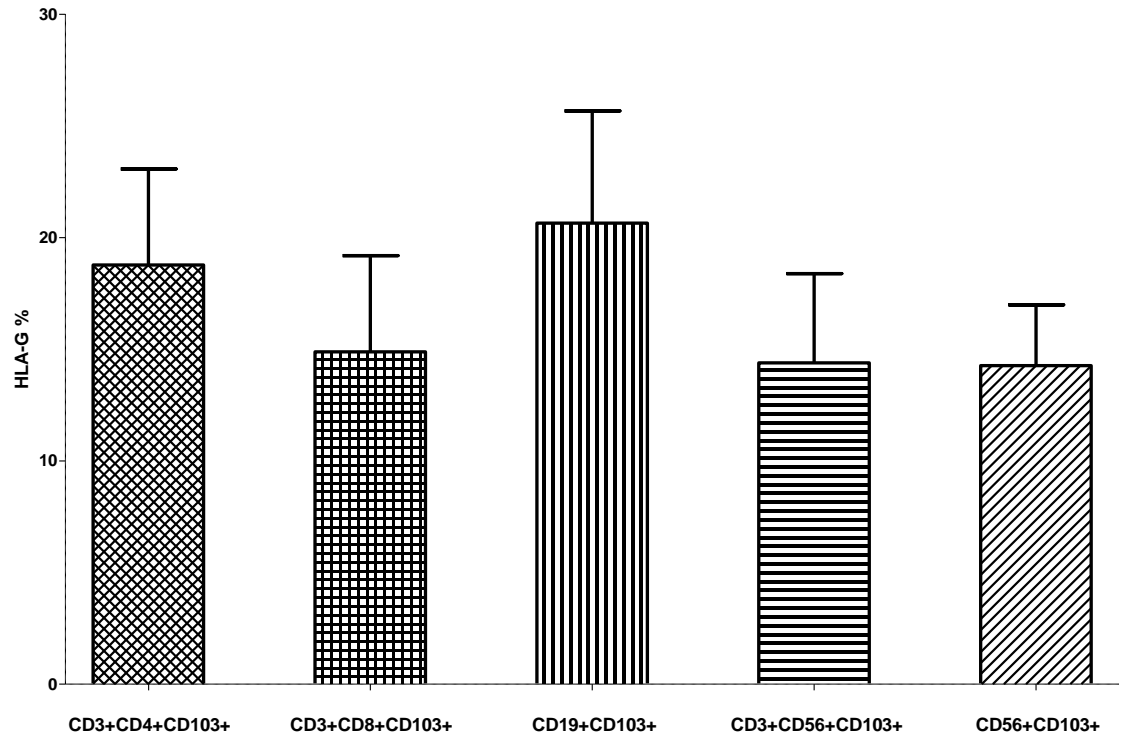


Fig. 5.21. HLA-G expressed by CD103+ PBMC populations after CMV induction for a week. Results are expressed as mean +/- SEM (n = 10).

5.22. Comparison of HLA-G expression by CD103+ PBMCs at rest and following CMV culture

After obtaining data from resting cells and after CMV induction, HLA-G expression on CD103+ PBMCs was compared (Fig. 5.22) between the tested population. When a two-tails Paired t test was performed to investigate the difference among the groups, significant differences were obtained in all the examined cells (CD3+CD+ P= 0.0020, CD3+CD8+ P= 0.0078, CD19+ P= 0.0034, CD3+CD56+ P= 0.0251 and CD56+ P= 0.0006).

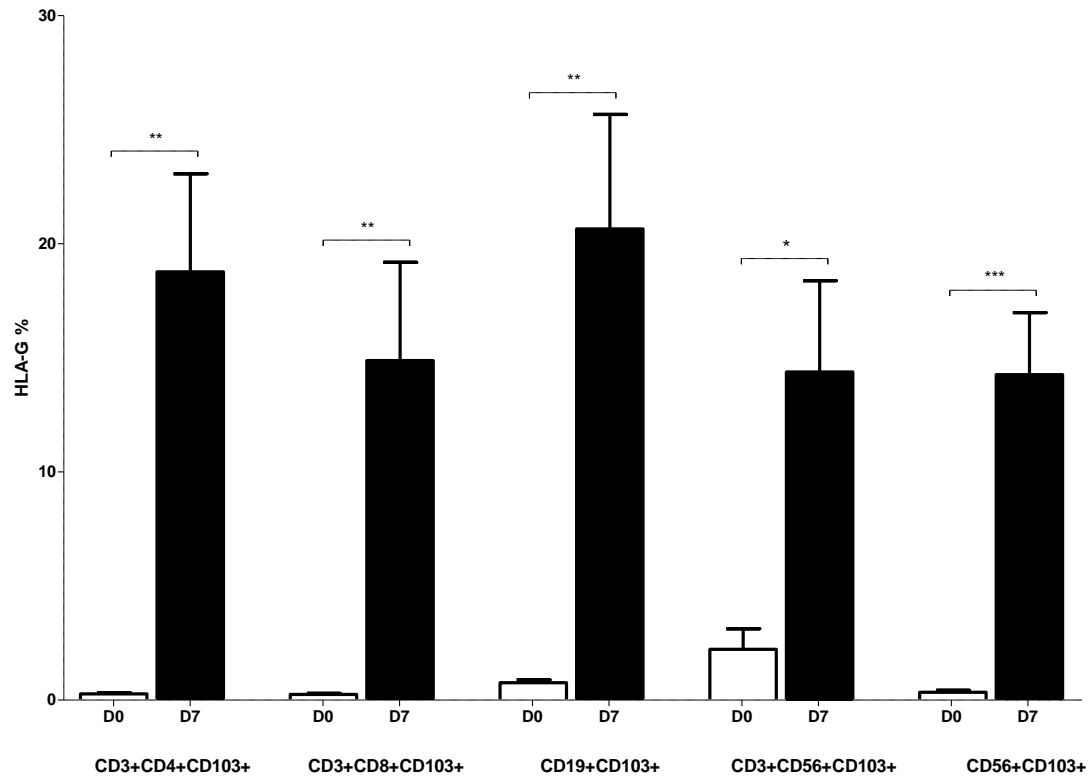


Fig. 5.22. Comparison of HLA-G expressed by freshly isolated CD103+ PBMC populations (empty columns) and CMV induced cells (black columns = D7). Results are expressed as mean \pm SEM (n = 10), *P<0.05, ***P<0.001.

5.23. Summary and discussion of CMV effects on CD103 expression

In brief, regardless CMV status of those healthy subjects involved in CD103 assessment, the expression of this receptor was significantly upregulated on the surface of CD3+CD8, CD3+CD56+ cell and CD56+ lymphocytes following stimulation with CMV antigens for a week. Similarly, when HLA-G was measured on CD103+ subsets, significant CC103+ cells were expressing HLA-G particularly CD3+CD4+ cells, CD3+CD8+lymphocytes, CD19+ cells, CD3+CD56+ ad NK cells Table 5.1.

Figure	Molecule	Cell population	CMV
5.19	CD103	CD3+CD8+	Not applicable
5.19	CD103	CD3+CD56+	Not applicable
5.19	CD103	CD56+	Not applicable
5.22	HLA-G+CD103+	CD3+CD4+	Not applicable
5.22	HLA-G+CD103+	CD3+CD8+	Not applicable
5.22	HLA-G+CD103+	CD19+	Not applicable
5.22	HLA-G+CD103+	CD3+CD56+	Not applicable
5.22	HLA-G+CD103+	CD56+	Not applicable

Table 5.1. Summary of significant differences in proportions of CD103 and HLA-G+CD103+ cells following induction with CMV antigens.

Regarding CD103 expression by peripheral blood PBMCs in healthy subjects, CD103 demonstrated very low on resting lymphocytes however, after induction with CMV proteins, significant upregulation of CD103 by CD8+ T lymphocytes and NK cells were recorded. CD103 expression has been shown to associate with in local regulatory memory T cells in salivary glands in murine CMV infected mice (C. J. Smith, Caldeira-Dantas, Turula, & Snyder, 2015). Infection with CMV can potentially induce the production of TGF- β 1 which was confirmed in the current study with qPCR, added to this, Michelson et al. have revealed that of TGF- β 1 could be upregulated in CMV infected fibroblasts (Michelson et al., 1994a). on the other hand, TGF- β can induce the and upregulate the expression of CD103 (D. Wang et al., 2004) in peripheral blood mononuclear cells which lead to induction of CD4+ T reg that have suppressive properties (W. Chen et al., 2003). Moreover, CD103 could play an important role in the migration of CD8+ lymphocytes to mucosal tissues (Laidlaw et al., 2014). This suggests the influence of CD103 on mobilising such regulatory cells and CTL to the CMV active mucosal tissues where such cells can impose immunosuppressive conditions, favouring viral replication and spread, or it could be a strategy of the immune system to recruit lymphocytes from peripheral circulation to act against viral replication in mucosal and endothelial tissues.

CHAPTER SIX

RESULTS

**Influence of CMV induction on BAFF and BAFF-
R expression**

B cell activation factor (BAFF) is an essential cytokine involved in B cell interaction with T cells (Craxton et al., 2003); this protein was found to increase during autoimmune diseases (Groom et al., 2002; J. Zhang et al., 2001), it could enhance Ab production and in transplant patients its elevated levels correlated with increased risk of rejection (Stegall et al., 2010). This protein has a specific ligand, BAFF-R, found on B cells (Bossen & Schneider, 2006) and plays a role in activating T cells and T reg (Mackay & Leung, 2006; Schneider, 2005). The results of Chapter 3 showed that B cells were one of the lymphocyte subsets showing the greatest increase in proportions of HLA-G expressing cells following stimulation with CMV antigens. It was therefore of interest to study the effects of CMV antigens on the B cell activating factor BAFF and its receptor. This section will describe the effects of CMV antigen stimulation on BAFF and BAFF-R expression by various PBMC populations from 9 healthy subjects (5 CMV+ and 4 CMV-). The cultured PBMCs were incubated for a week with or without viral antigens and at day seven of the experiment, the PBMCs were harvested and phenotyped with monoclonal antibodies specific for the two assessed proteins. In addition, expression of HLA-G was evaluated on those PBMCs displaying BAFF and BAFF-R proteins following culture with and without CMV antigen. Data were acquired with an Accuri C6 flow cytometry and analysed with Accuri C6 software.

6.1. Expression of BAFF in PBMC populations after CMV stimulation in CMV+ subjects

The BAFF receptor was assessed on the surface of various PBMC populations following culture with CMV antigens for a week. Fig. 6.1a exhibits some flow cytometry acquisition dot plots for BAFF and BAFF-R expression on lymphocytes. The cell types with highest proportions of cell surface BAFF antigen in response to CMV induction were CD56+ lymphocytes (10.9%) followed by CD14+HLA-DR+ cells (10.8%), then CD19+ B cells (10.6%) and CD3+CD56+ T cells (10.1%). The remaining groups (CD3+CD8+ and CD3+CD4+ cells) had proportions of 3.04% and 1.95% respectively). No statistically significant difference was noted between the cell populations when a one-way ANOVA (Tukey's Multiple Comparison Test) was applied ($P=0.4885$) (Fig. 6.1b).

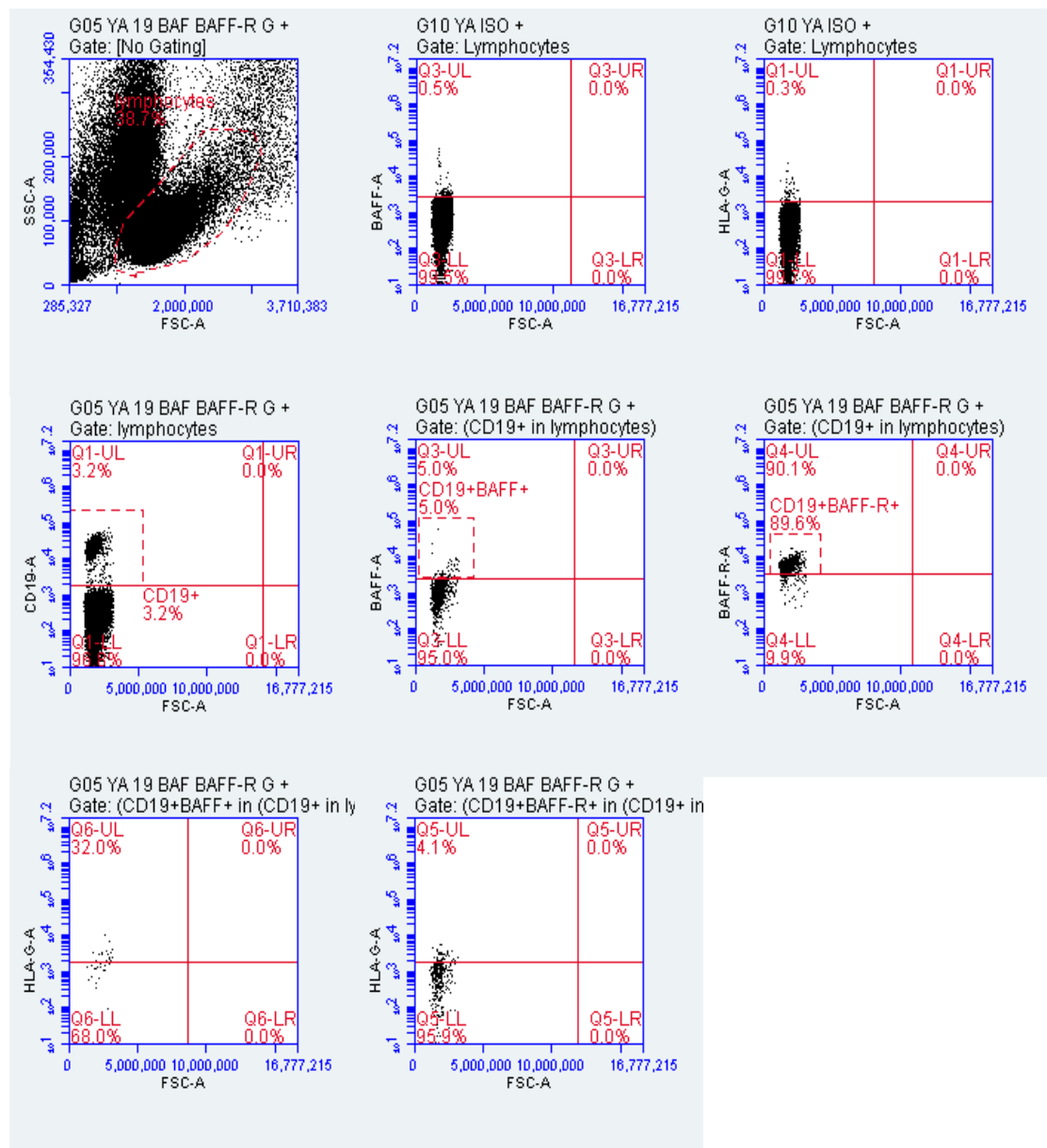


Fig. 6.1a. Acquisition plots of Accuri C6 flow cytometry exhibiting in order: scatter profile showing lymphocyte gate; isotype control, CD19, BAFF, BAFF-R and HLA-G co-labelling showing gates used for BAFF and BAFF-R proportions on CD19+ cells after culture with CMV antigens.

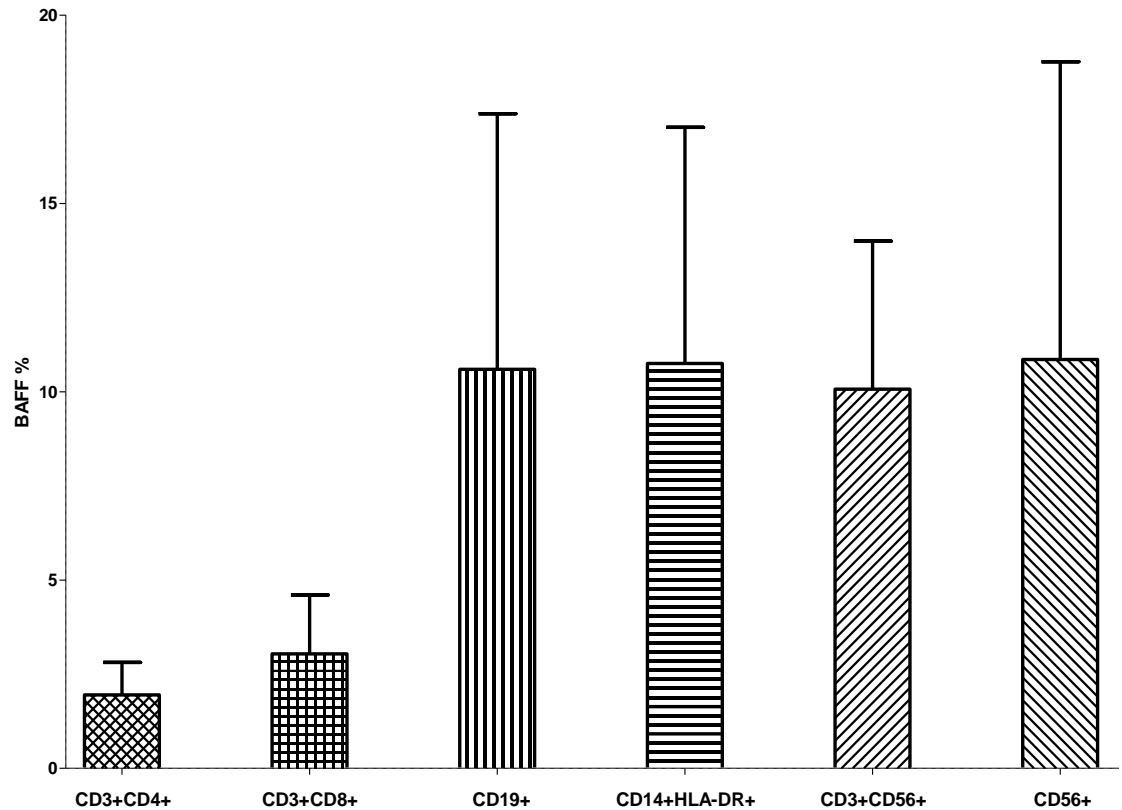


Fig. 6.1b. BAFF expression on PBMC populations following culture with CMV antigen in CMV + individuals. Results are expressed as mean \pm SEM (n = 5). No statistically significant differences were found between the tested groups $P > 0.05$.

6.2. Expression of BAFF in control PBMCs after culture in CMV+ subjects

The expression of BAFF antigen on the control non-stimulated PBMCs from healthy subjects was also evaluated on various PBMC groups (Fig. 6.2). The cell types with highest proportion of BAFF+ expression was CD14+HLA-DR+ monocytes (4.6%). Proportions of BAFF antigen for the rest of the groups were: CD3+CD56+cells (1.1%) or less than 1% (CD19+=0.8%, CD56+=0.4%, CD3+CD8+=0.22% and CD3+CD4+=0.1%). Upon performing a one-way ANOVA (Tukey's Multiple Comparison Test), there was no statistical difference between the groups ($P=0.4374$).

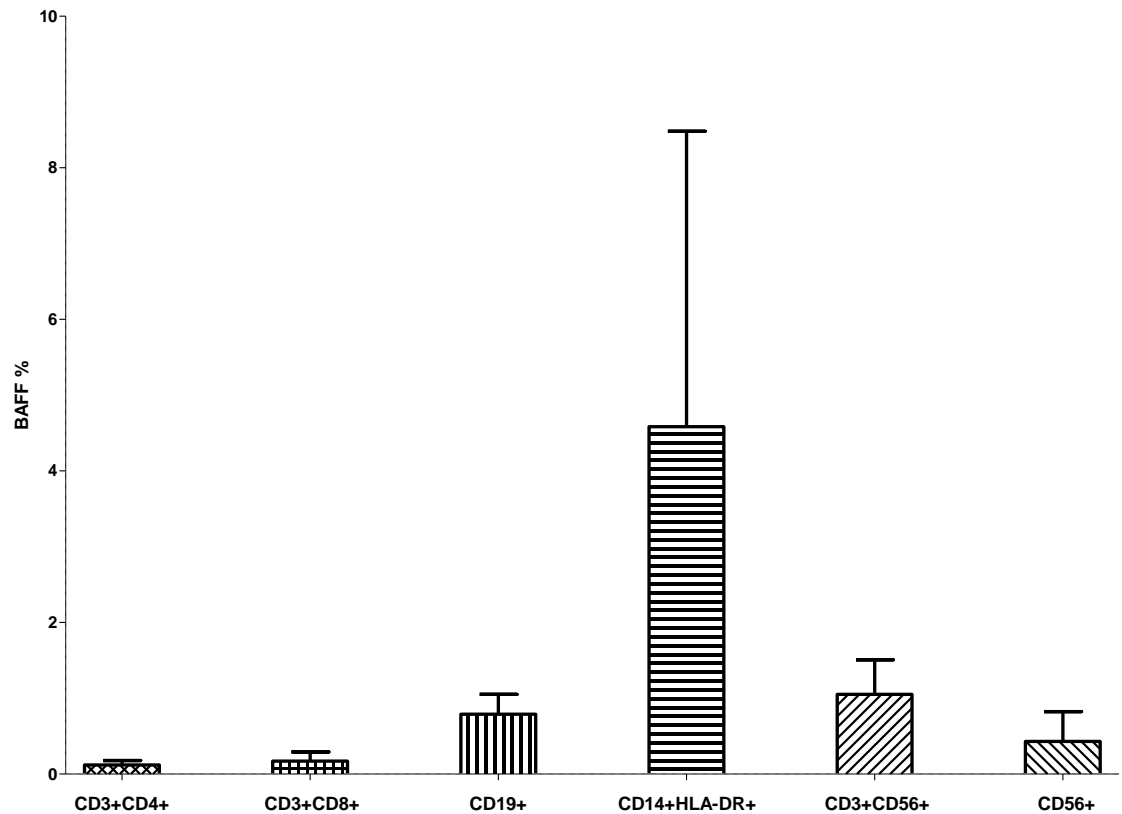


Fig. 6.2. BAFF expression on PBMC populations following culture without CMV antigen in CMV+ individuals. Results are expressed as mean \pm SEM ($n = 5$). No statistically significant differences were found between the groups $P > 0.05$.

6.3. Comparison of BAFF expression after culture with or without CMV stimulation in CMV + subjects

The proportions of PBMCs expressing BAFF antigen following culture with CMV antigens for a week were then compared between control non-stimulated and stimulated cells (Fig. 6.3). Overall, every cell subset expressed higher proportions of BAFF in response to CMV antigen but the only cell populations that exhibited a statistically significant increase were CD3+CD8+ lymphocytes ($P=0.0313$) and CD3+CD56+ cells ($P=0.0313$). The remaining group of cells showed no significant differences between the induced and non-induced groups ($P > 0.05$) when a Wilcoxon matched-pairs signed rank test (two tail) was performed. However, a close to significant level was noted in CD3+CD4+ lymphocytes ($P=0.062$), CD19+ cells ($P=0.06$) and CD56+ lymphocytes ($P=0.0625$).

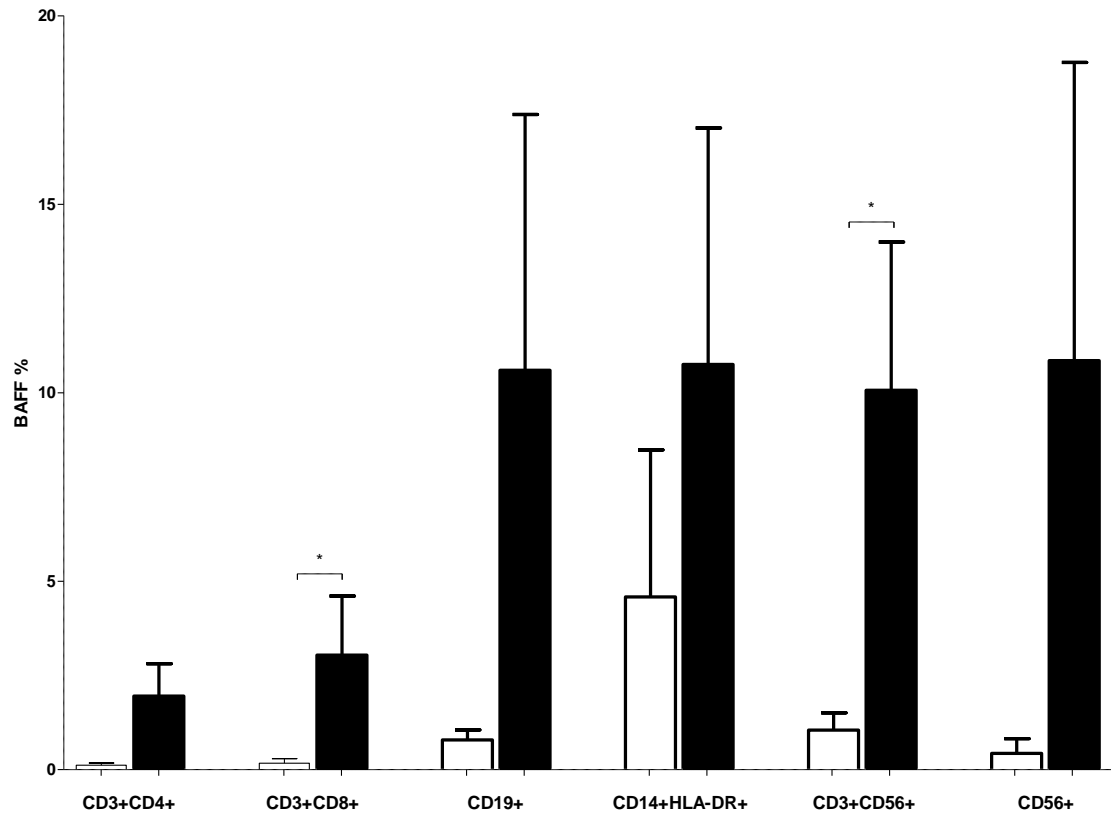


Fig. 6.3. Comparison of BAFF expression on PBMC populations following culture with or without CMV antigen in CMV+ individuals, empty columns (non-stimulated, n = 5) filled columns (stimulated, n = 5). Results are expressed as mean \pm SEM, *P<0.05.

6.4. Expression of HLA-G on BAFF+ PBMCs after culture in CMV+ subjects

HLA-G expression was also evaluated on those PBMCs that expressed BAFF antigen. As previously mentioned in Chapter 2, data were acquired using an Accuri C6 flow cytometer after channel compensation for three and four colours (Fig. 6.4). Proportions of HLA-G+ cells were increased in almost all the groups apart from CD14+HLA-DR+ monocytes (3.7%), while the lymphocyte populations had increasing proportions of HLA-G+ cells (CD3+CD4+=17.8%, CD3+CD8+=17.8%, CD3+CD56+=30.2%, CD19+=30.8%, and CD56+=42.1%). There was no statistically significant difference among the compared groups when a one-way ANOVA (Tukey's Multiple Comparison Test) was performed (P=0.0923).

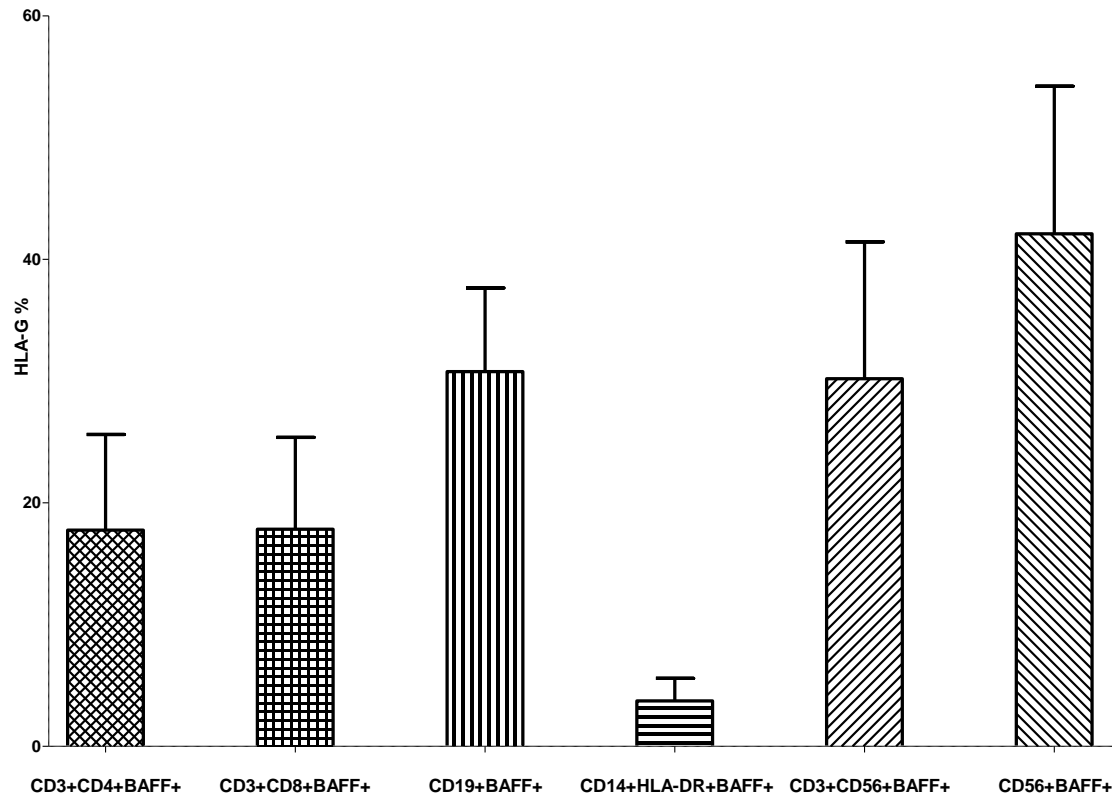


Fig. 6.4. The expression of HLA-G on BAFF+ PBMCs following culture with CMV antigen in CMV+ individuals. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

6.5. Expression of HLA-G on BAFF+ mononuclear cells in control CMV+ subjects

In the control non-stimulated samples after incubating the cells for seven days, the expression of HLA-G was evaluated on PBMC populations that expressed the BAFF protein (Fig. 6.5). The proportions of BAFF+HLA-G+ cells in almost all the tested groups were below 2%, with CD19+ cells being the highest cell expressed HLA-G (1.9%) and the lowest proportion was displayed by CD14+HLA-DR+ cells (0.04%). The proportion of HLA-G expression was in the following descending order: CD3+CD8+ (0.7%), CD3+CD56+ (0.62%), CD3+CD4+ (0.34%) and CD56+ (0.28%). In order to compare the mean proportions of HLA-G+ cells between various groups, a one-way ANOVA (Tukey's Multiple Comparison Test) was applied and statistical significant difference noted with overall $P = 0.0490$. Specifically,

significant difference was obtained between CD19+ cells vs CD14+HLA-DR+ cells (*P<0.05).

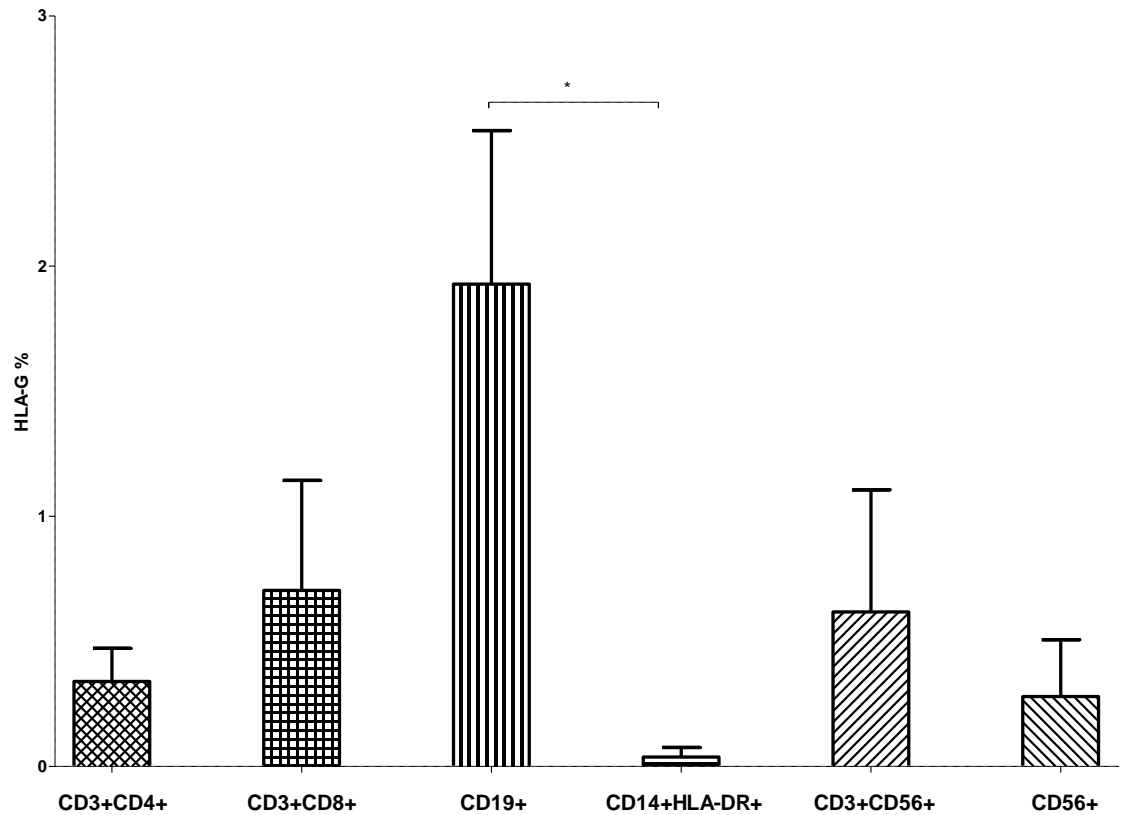


Fig. 6.5. The expression of HLA-G on BAFF + PBMCs following culture without CMV antigen in CMV + individuals. Results are expressed as mean \pm SEM, $n = 5$, *P<0.05.

6.6. Comparison of HLA-G expression on BAFF+ PBMCs in control and stimulated cells in CMV + subjects

In order to evaluate the difference between the expression of HLA-G on BAFF+ PBMCs after culture with and without CMV antigens, a comparison was made (Fig. 6.6) using a Paired t test (two tailed). Significant differences were obtained in almost all the compared set of cells (CD3+CD4+ P=0.0438, CD3+CD8+ P=0.0380, CD19+ P=0.0070, CD3+CD56+ P=0.0299 and CD56+ P=0.0131). Only CD14+HLA-DR+ cells did not show statistical difference with P=0.0666.

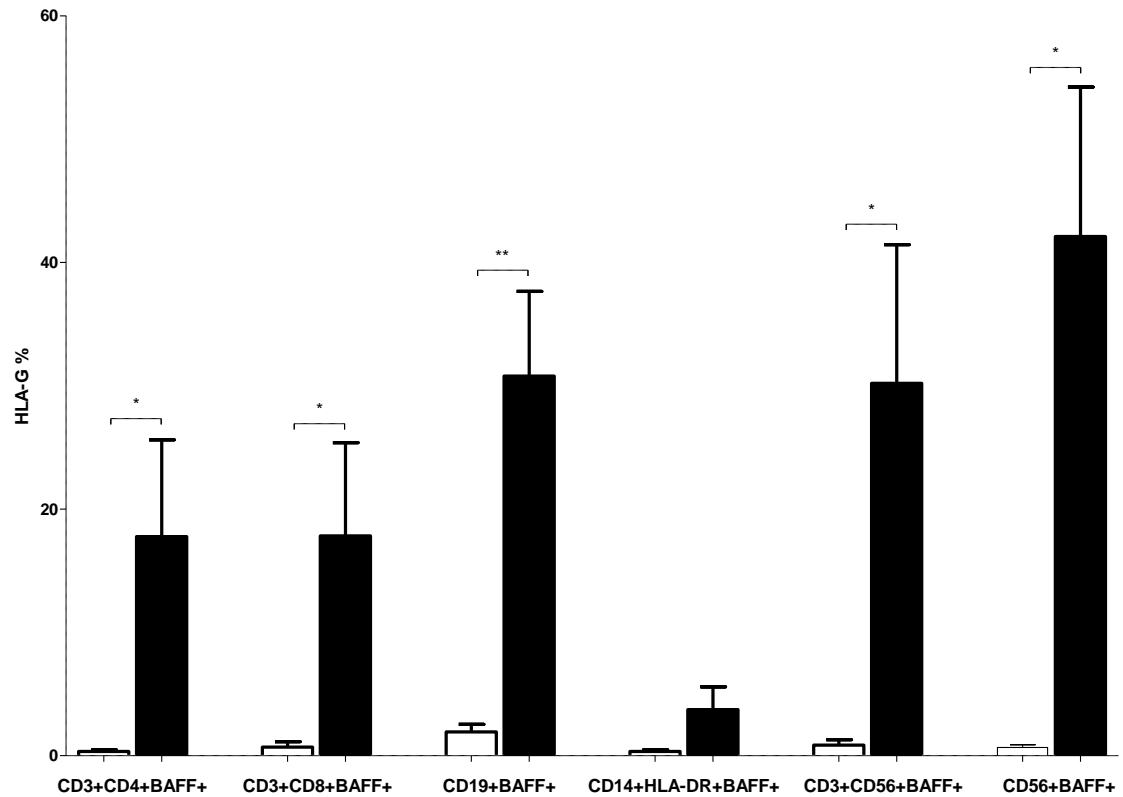


Fig. 6.6. Comparison of HLA-G expression on BAFF+ PBMCs following culture with and without CMV antigen in CMV+ individuals. Empty columns (non-stimulated, n = 5), filled columns (stimulated, n = 5). Results are expressed as mean \pm SEM, * $P < 0.05$, ** $P < 0.01$.

6.7. Expression of BAFF-R in cultured PBMCs with CMV antigen in CMV+ subjects

In the same way, BAFF-R expression was also assessed on mononuclear cells from healthy subjects stimulated with CMV antigens for seven days (Fig. 6.7). A high proportion of CD19+ cells (82.2%) expressed this molecule and the proportion of the BAFF-R+ cells was lowest on CD3+CD56+ lymphocytes (11.9%). The remaining subpopulations expressed the molecule in the following descending order: CD3+CD8+ (34.6%), CD56+ (24.7%), CD3+CD4+ (15.9%) and CD14+HLA-DR+ (15.2%). A comparison among the groups was made with a one-way ANOVA (Tukey's Multiple Comparison Test) and this was significant with overall $P = 0.0001$. In particular, significant differences were obtained between CD3+CD4+ vs CD19+ (** $P < 0.01$), CD3+CD8+ vs CD19+ (** $P < 0.05$), CD19+ vs CD14+HLA-DR+

(***P<0.01), CD19+ vs CD3+CD56+ (***P<0.01) and CD19+ vs CD56+ (***P<0.05) cells.

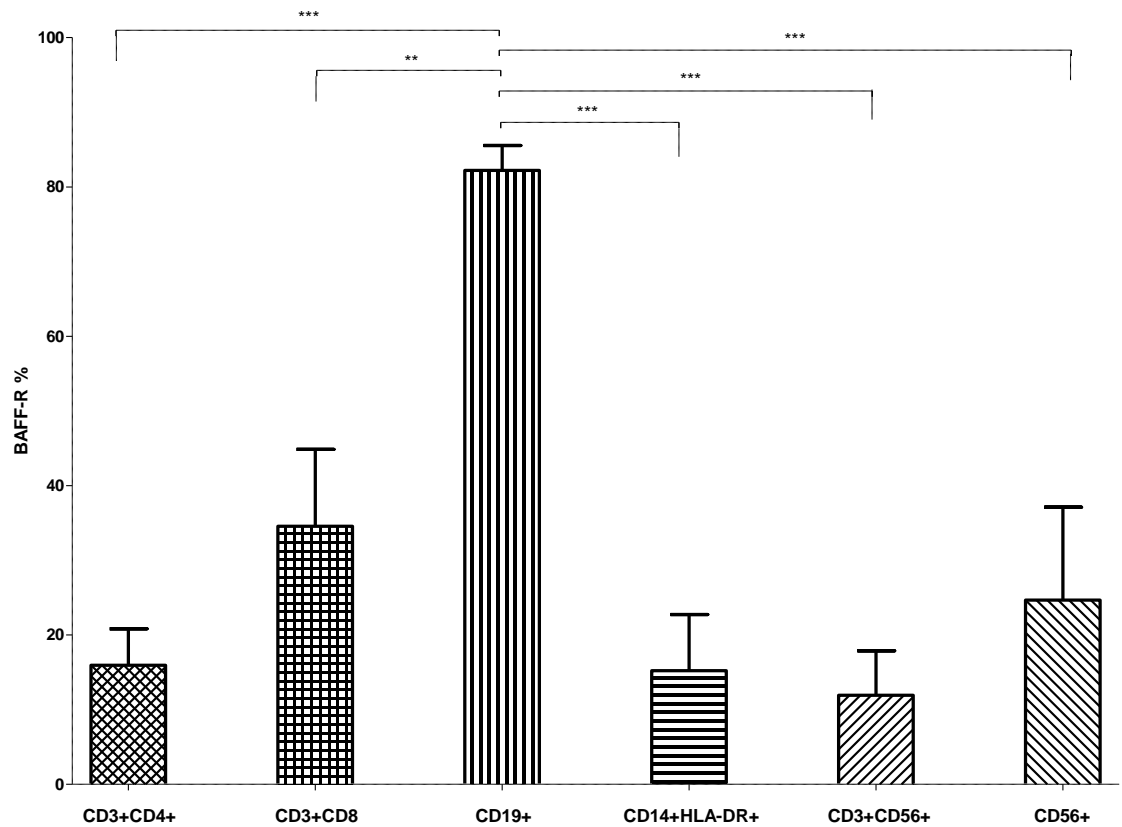


Fig. 6.7. The expression of BAFF-R on various PBMC populations following culture with CMV antigen in CMV+ individuals. Results are expressed as mean \pm SEM, n = 5, **P<0.01, ***P<0.001.

6.8. Expression of BAFF-R in control cultured PBMCs from CMV+ subjects

In the control non-stimulated PBMCs from healthy CMV+ individuals, after a week of cell incubation, BAFF-R was also measured on various cell groups (Fig. 6.8). The proportion of BAFF on CD19+ lymphocytes was highest (96.7%), while the other cells populations showed variable proportions (CD3+CD8+=38.9%, CD14+HLA-DR+=14.6%, CD56+=12.1%, CD8+=10.7%, CD3+CD4+=8.25%, and CD3+CD56+=1.5%). A comparison between the groups was performed with a one-way ANOVA (Tukey's Multiple Comparison Test) and statistically significant

difference was noted with overall $P=0.0001$. Particularly, the significant differences between the PBMC populations are summarised in Table 6.1.

Tukey's Multiple Comparison Test	Significant? $P < 0.05$?
CD3+CD4+ vs CD3+CD8+	**$P<0.01$
CD3+CD4+ vs CD19+	***$P<0.001$
CD3+CD4+ vs CD14+HLA-DR+	ns
CD3+CD4+ vs CD3+CD56+	ns
CD3+CD4+ vs CD56+	ns
CD3+CD8+ vs CD19+	***$P<0.001$
CD3+CD8+ vs CD14+HLA-DR+	*$P<0.05$
CD3+CD8+ vs CD3+CD56+	***$P<0.001$
CD3+CD8+ vs CD56+	*$P<0.05$
CD19+ vs CD14+HLA-DR+	***$P<0.001$
CD19+ vs CD3+CD56+	***$P<0.001$
CD19+ vs CD56+	***$P<0.01$
CD14+HLA-DR+ vs CD3+CD56+	ns
CD14+HLA-DR+ vs CD56+	ns
CD3+CD56+ vs CD56+	ns

Table. 6.1. One-way ANOVA test comparing the expression of BAFF-R on various PBMC populations after cell culture.

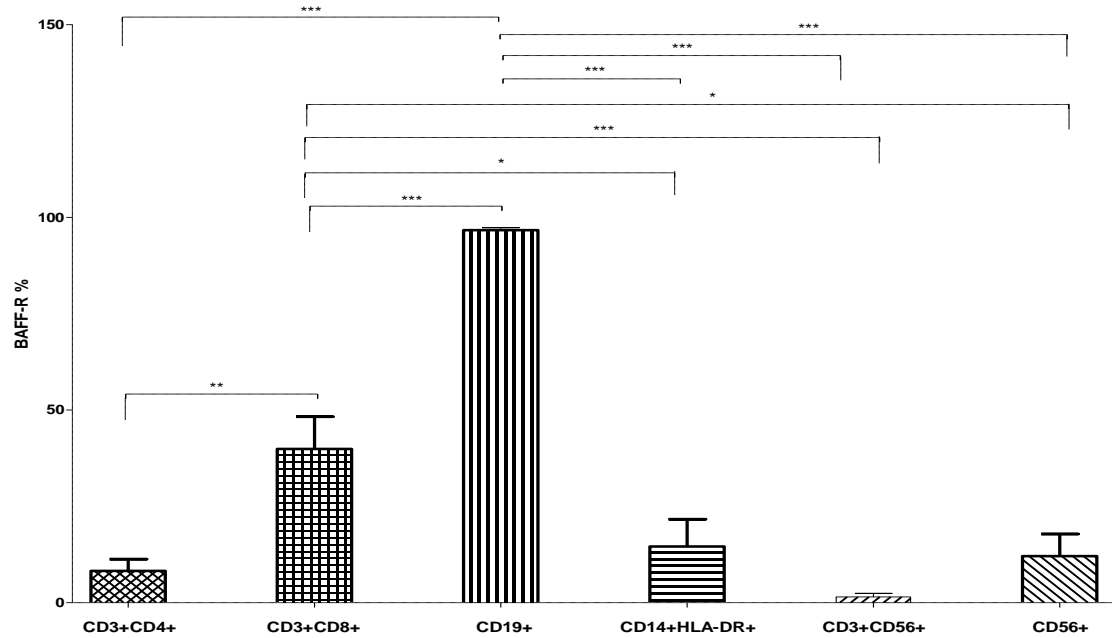


Fig. 6.8. The expression of BAFF-R on various PBMC populations following culture without CMV antigen in CMV+ individuals. Results are expressed as mean +/- SEM, n = 5, * P<0.05, ** P<0.01, ***P<0.01.

6.9. Comparison of BAFF-R expression with and without CMV antigen stimulation in CMV+ individuals

In CMV+ subjects, proportions of cells expressing the BAFF-R were compared between the stimulated and control cultured PBMCs after a week of incubation (Fig. 6.9). When the groups were compared using a two tailed Wilcoxon matched-pairs signed rank test, no statistical difference was seen, however, some of the populations showed P value close to significant level (CD19+ lymphocytes, P= 0.0625 and CD3+CD56+ cells, P=0.0625).

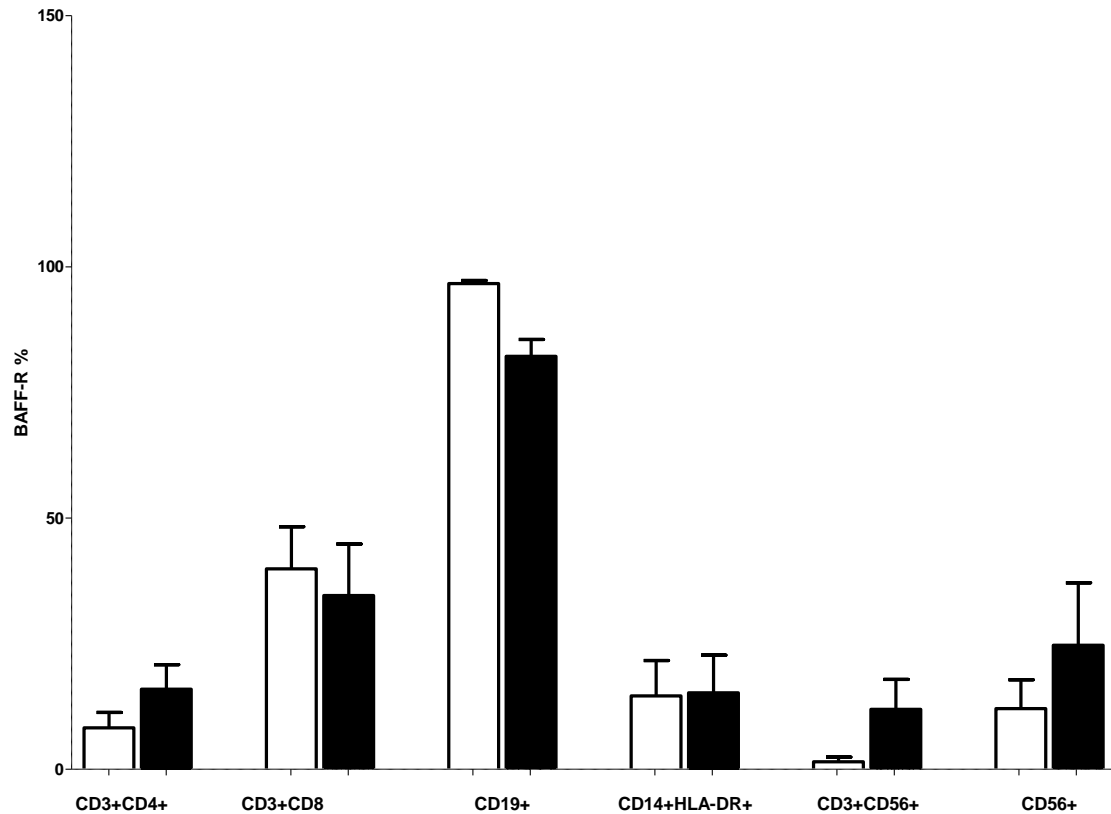


Fig. 6.9. Comparison of BAFF-R expression on various PBMC populations following culture with and without CMV antigen in CMV+ subjects. Empty columns (non-stimulated, n = 5), filled columns (stimulated, n = 5). Results are expressed as mean \pm SEM, no statistically significant differences were noted $P > 0.05$.

6.10. Expression of HLA-G on BAFF-R+ PBMCs cultured with CMV antigen in CMV+ subjects

HLA-G cell surface expression was assessed in those PBMC groups expressing the BAFF-R antigen, as depicted in Fig. 6.10. The proportions of HLA-G+ cells among CD3+CD56+ lymphocytes were highest (46.8%), while the proportion was lowest (3.2%) in CD14+HLA-DR+ cells. The proportion of cells expressing HLA-G on the remaining BAFF-R+ subsets was in the following increasing order: CD19+=6.4%, CD3+CD8+=7.9%, CD3+CD4+=10.7% and CD56+=23.0%. When a one-way ANOVA (Tukey's Multiple Comparison Test) was performed, there was statistically significant difference ($P=0.0001$). The statistical significance of differences between the groups are summarised in Table 6.2.

Tukey's Multiple Comparison Test	Significant? P < 0.05?
CD3+CD4+ vs CD3+CD8+	ns
CD3+CD4+ vs CD19+	ns
CD3+CD4+ vs CD14+HLA-DR+	ns
CD3+CD4+ vs CD3+CD56+	***P<0.001
CD3+CD4+ vs CD56+	ns
CD3+CD8+ vs CD19+	ns
CD3+CD8+ vs CD14+HLA-DR+	ns
CD3+CD8+ vs CD3+CD56+	***P<0.001
CD3+CD8+ vs CD56+	ns
CD19+ vs CD14+HLA-DR+	ns
CD19+ vs CD3+CD56+	***P<0.001
CD19+ vs CD56+	ns
CD14+HLA-DR+ vs CD3+CD56+	***P<0.001
CD14+HLA-DR+ vs CD56+	*P<0.05
CD3+CD56+ vs CD56+	**P<0.01

Table 6.2. One-way ANOVA test comparing the expression of BAFF-R on various PBMC populations after cell culture.

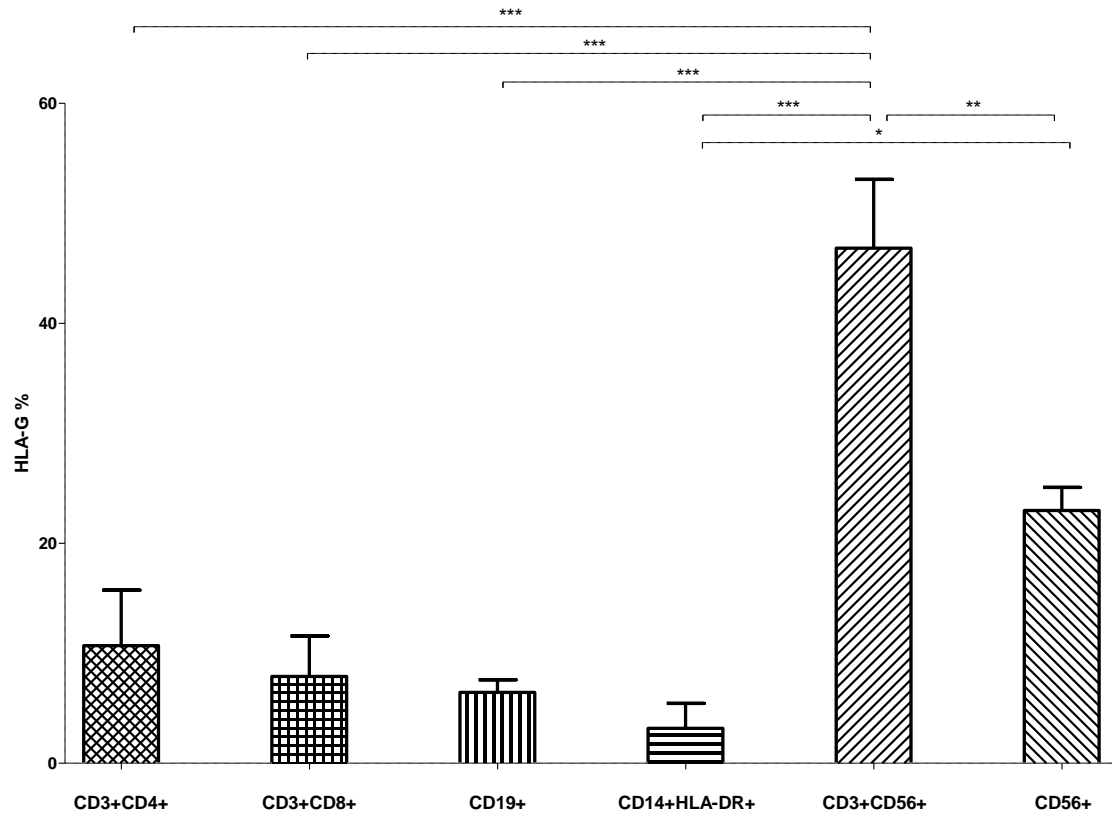


Fig. 6.10. The expression of HLA-G on BAFF-R + PBMC populations following culture with CMV antigen in CMV + subjects. Results are expressed as mean \pm SEM, $n = 5$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (ANOVA).

6.11. Expression of HLA-G on PBMCs expressing BAFF-R in control CMV+ subjects

In control PBMCs from healthy subjects, HLA-G was measured on cells expressing BAFF-R protein following culture without CMV antigens (Fig. 6.11). Overall, the proportion of HLA-G expressing cells was less than 2% with highest percentage (1.9%) on CD3+CD56+ lymphocytes and lowest proportion displayed by CD14+HLA-DR+ cells (0.2%). The proportion of HLA-G+ cells in the rest of the subsets were in the following descending order: CD19+=0.97%, CD3+CD4+=0.95%, CD56+=0.73%, CD3+CD8+=0.57%. Upon comparing the groups with a one-way ANOVA (Tukey's Multiple Comparison Test), there was no statistically significant difference among the tested populations, ($P = 0.1440$).

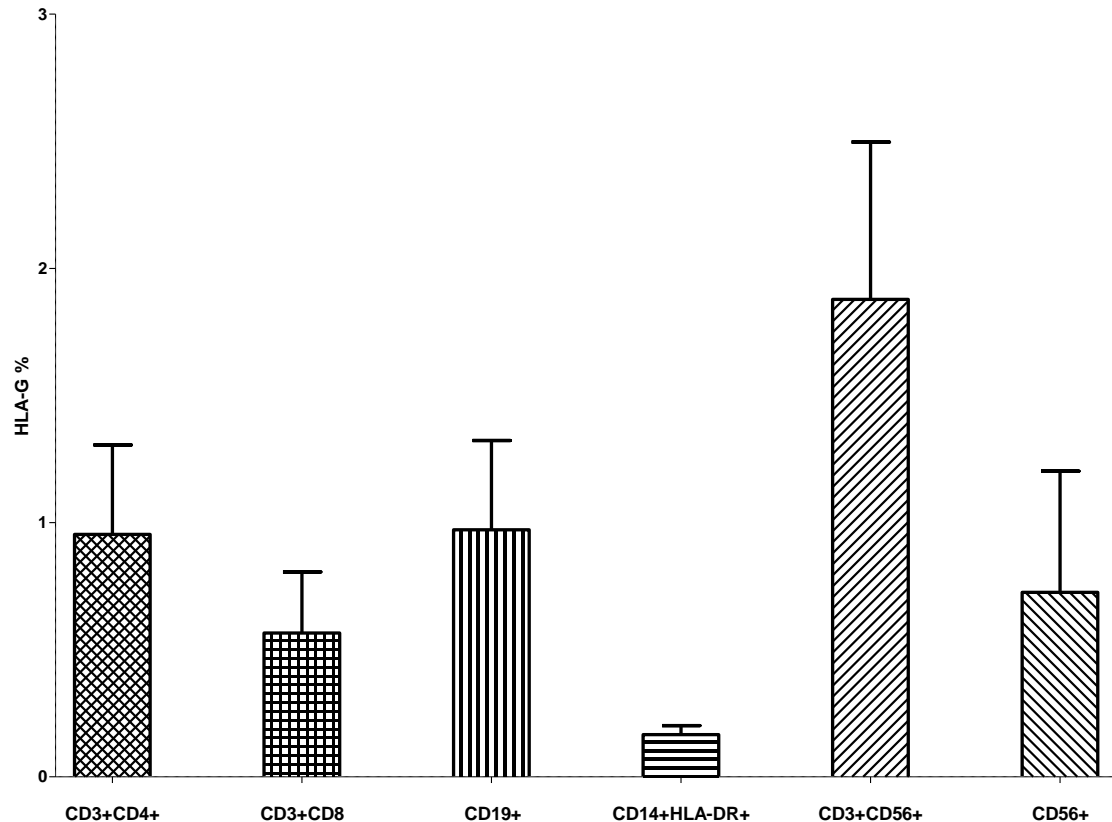


Fig. 6.11. Proportions of cells expressing HLA-G in BAFF-R+ PBMC populations following culture without CMV antigen in CMV+ subjects. Results are expressed as mean \pm SEM, $n = 5$, no statistically significant differences were noted.

6.12. Comparison of HLA-G expression on BAFF-R+ PBMCs in control and induced cells in CMV+ subjects

HLA-G expression on various mononuclear cells was then compared between control and CMV antigen-stimulated cells (Fig. 6.12). Most of the tested populations exhibited significantly higher proportions of HLA-G following CMV culture. When a two tailed Paired t test was performed, there were statistically significant differences in the means of CD19+ cells ($P=0.0013$), CD3+CD56+ lymphocytes ($P=0.0007$) and CD3-CD56+ cells ($P=0.0003$). No statistically significant differences were noted ($P>0.05$) for CD14+HLA-DR+ cells and the P values for CD3+CD4+ cells and CD3+CD8+ cells were close to significant levels ($P= 0.0549$ and $P=0.0601$).

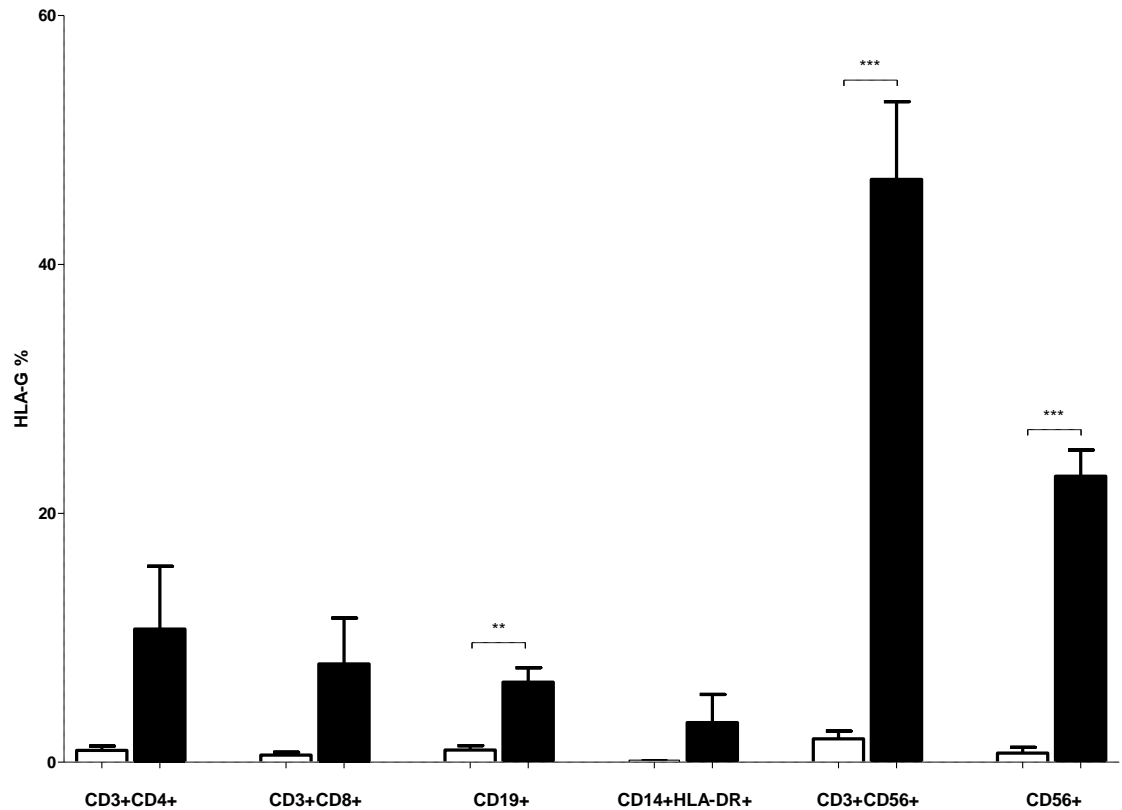


Fig. 6.12. The expression of Proportions of cells expressing HLA-G for BAFF-R + PBMC populations following culture with and without CMV antigen in CMV + subjects. Filled columns (non-stimulated, n = 5), empty columns (stimulated, n = 5). Results are expressed as mean +/- SEM, ***P<0.01, **P<0.05.

6.13. Expression of BAFF on PBMC populations after culture with CMV antigen in CMV- subjects

BAFF antigen expression was evaluated on various PBMC populations in CMV- subjects in response to CMV induction and after a week of cell culture the proportion of positive cells determined using C6 flow cytometry (Fig. 6.13). The proportions of cells expressing the molecule was notably elevated on CD3+CD56+ cells (21.15%), while only 0.48% of CD3+CD4+ lymphocytes expressed BAFF. The other mononuclear cells tested expressed the protein in descending order: CD3+CD8+=1.11%, CD14+HLA-DR+=2.82%, CD56+=4.46% and CD19+=5.53%. The groups expressing the BAFF molecule were compared using a one-way ANOVA (Tukey's Multiple Comparison Test) and statistically significant differences among them was obtained with overall P= 0.0373. The significant differences were

recorded between CD3+CD4+ vs CD3+CD56+ (*P<0.05) and CD3+CD8+ vs CD3+CD56+ (*P<0.05) cells.

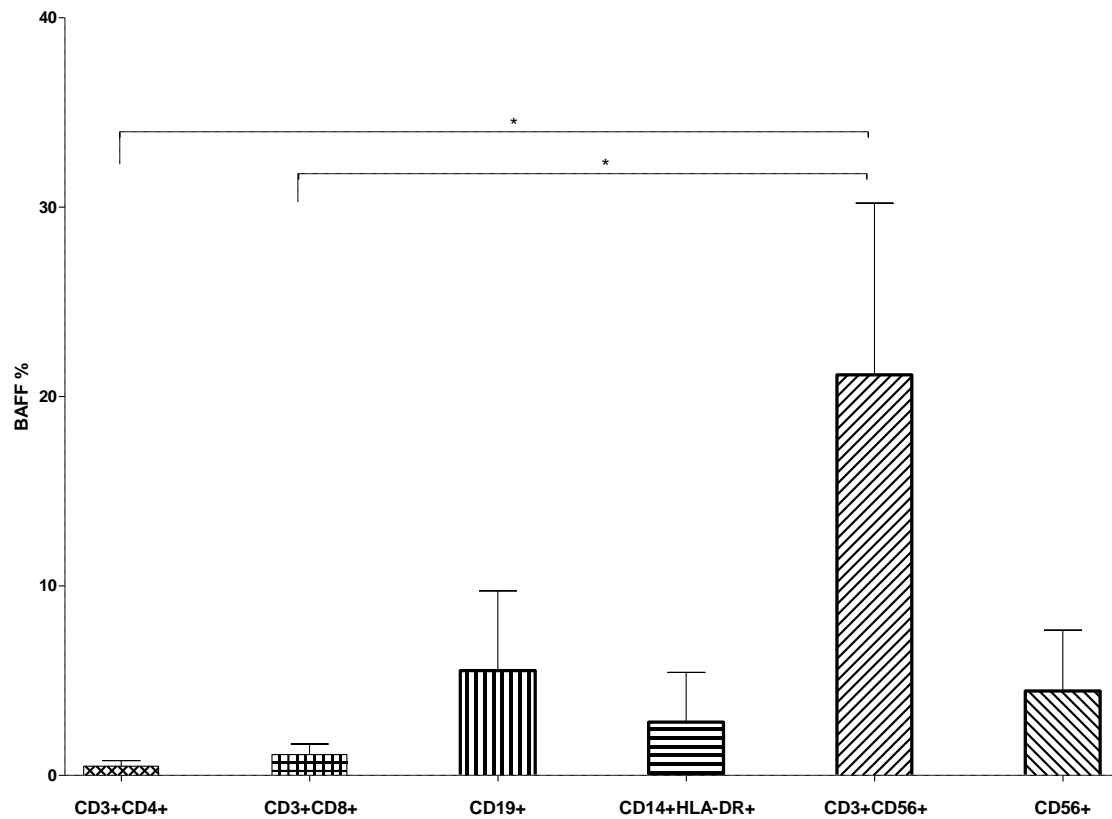


Fig. 6.13. Proportions of BAFF expressing cells from different PBMC populations following culture with CMV antigen in CMV- subjects. Results are expressed as mean \pm SEM, $n = 4$, * $P < 0.05$.

6.14. Expression of BAFF in control PBMCs after culture in CMV- subjects

In parallel with stimulated cells, the non-stimulated PBMCs were utilised to evaluate the level of BAFF antigen on various PBMC groups (Fig. 6.14). CD3+CD56+ lymphocytes were the most prominent cells that expressed the BAFF antigen with 14.2%, whereas very low proportions were recorded on the other populations (CD19+=2.2%, CD14+HLA-DR+=0.65%, CD3+CD8+=0.4%, CD56+=0.2% and CD3+CD4+=0.1%). The groups showed significant statistical differences with overall $P = 0.0158$ when a one-way ANOVA (Tukey's Multiple Comparison Test) was performed. Statistical significance was obtained between CD3+CD4+ vs

CD3+CD56+ (*P<0.05), CD3+CD8+ vs CD3+CD56+ (*P<0.05), CD14+HLA-DR+ vs CD3+CD56+ (*P<0.05) and CD3+CD56+ vs CD56+ (*P<0.05) cells.

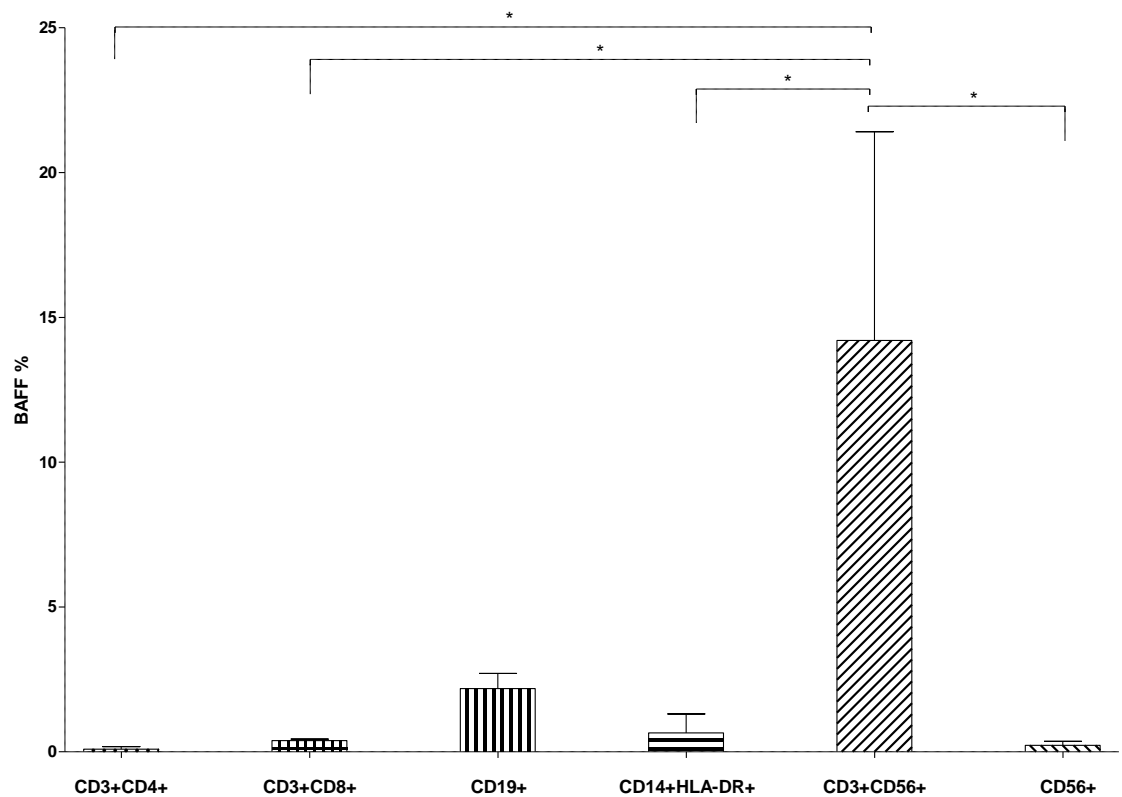


Fig. 6.14. BAFF expression on PBMC populations following culture without CMV antigen in CMV - subjects. Results are expressed as mean +/- SEM, n = 4, *P<0.05 (ANOVA).

6.15. Comparison of BAFF expression on PBMCs after culture with and without CMV antigen in CMV- subjects

For the purpose of determining the effects of the CMV antigen on the stimulated PBMCs, a comparison between the stimulated and non-stimulated cells was made using a two tail Wilcoxon matched-pairs signed rank test. None of the tested groups showed any statistically significant differences ($P>0.05$) upon cell induction despite the elevated proportions of BAFF antigen expressed by most of the stimulated mononuclear populations (Fig. 6.15). However, there was a close to significant difference in CD56+ cells ($P=0.0625$).

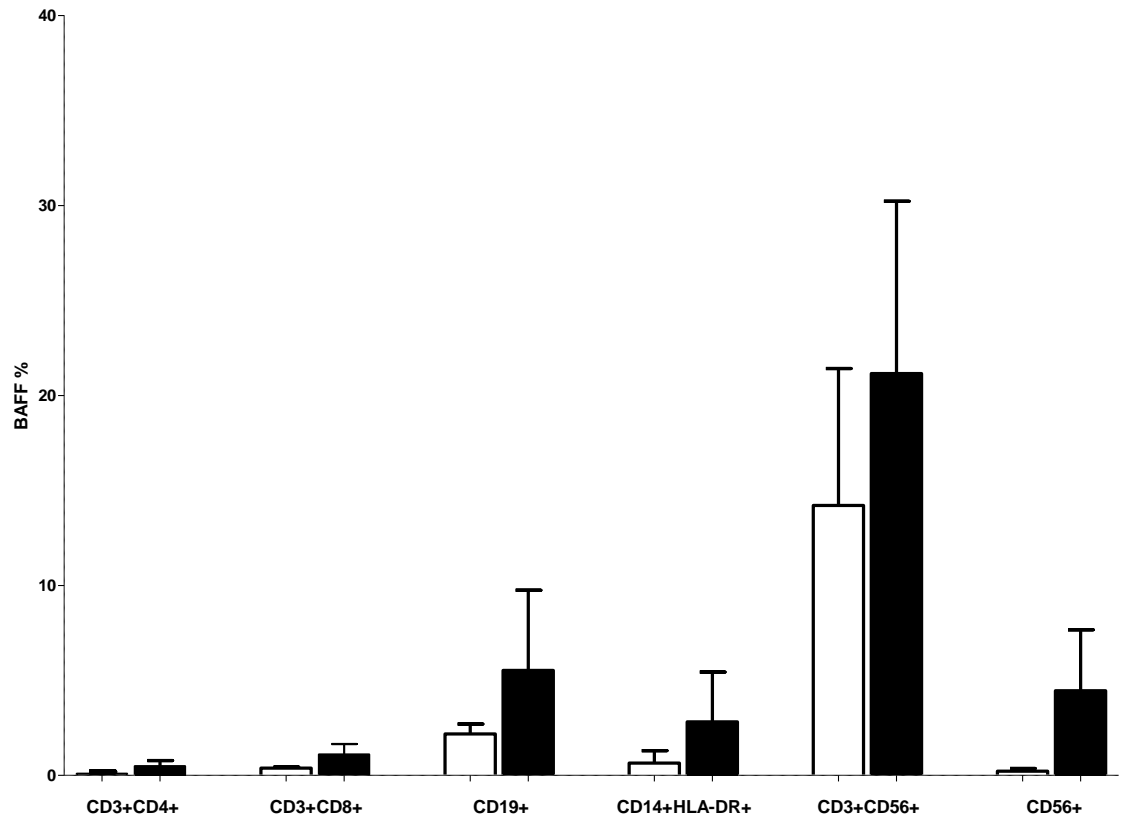


Fig. 6.15. Comparison of BAFF expression on PBMC populations following culture with and without CMV antigen in CMV⁻ individuals, empty columns (non-stimulated, n = 4) filled columns (stimulated, n = 4). Results are expressed as mean \pm SEM, no statistically significant differences were recorded between the groups.

6.16. Expression of HLA-G on BAFF⁺ PBMCs in response to CMV induction in CMV⁻ subjects

The same pattern was followed in CMV⁻ subjects, the proportions of HLA-G expressing cells was assessed on those mononuclear cells expressing BAFF molecule (Fig. 6.16). Most of the stimulated BAFF⁺ PBMCs expressed HLA-G in relatively high proportions, the highest percentage being in CD56⁺ cells (55.7%), while CD14+HLA-DR⁺ monocytes expressed the lowest proportion (3%) among the groups. The remaining cells had the following descending proportions: CD3+CD4⁺ (49.7%), CD3+CD56⁺ (49.3%), CD19⁺ (35.5) and CD3+CD8⁺ cells (35.3%). The groups were compared with a one-way ANOVA (Tukey's Multiple Comparison Test) and there were no statistically significant differences among them ($P=0.0607$).

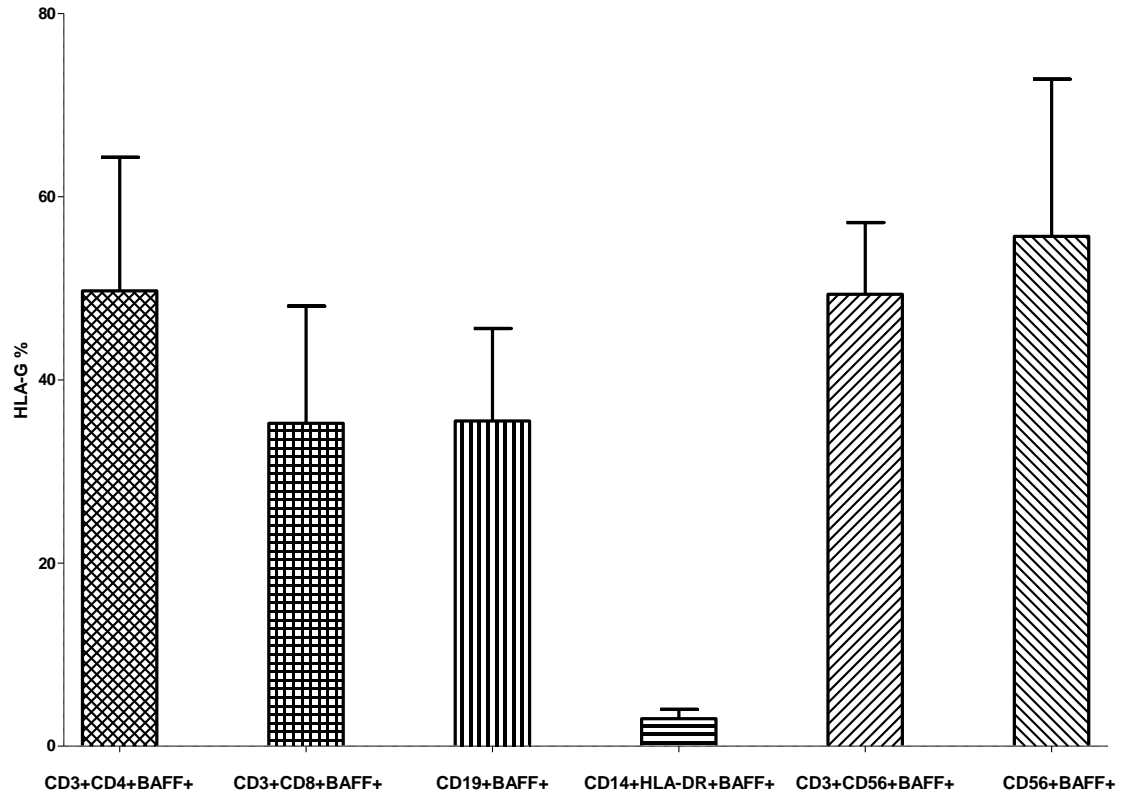


Fig. 6.16. The expression of HLA-G on BAFF+ PBMCs following culture with CMV antigen in CMV- individuals. Results are expressed as mean % positive cells \pm SEM, $n = 4$, $P > 0.05$.

6.17. Expression of HLA-G on BAFF+ PBMCs in control group

In the control non-stimulated PBMCs from CMV- subjects and following cell culture without CMV antigen, HLA-G was assessed on those mononuclear cells expressing the BAFF molecule (Fig. 6.17). In general, CD19+ cells were the highest expresser of HLA-G (4.93%), whereas, CD14+HLA-DR+ populations were the lowest expressers (0.69%). The remaining cells had the following descending proportions: CD3+CD56+ (2.6%), CD3+CD8+ (2.3%), CD56+ (1.0%) and CD3+CD4+ cells (0.9%). In order to compare the differences between the groups, a one-way ANOVA (Tukey's Multiple Comparison Test) was applied and it showed statistically significant difference among them with overall $P = 0.0049$. The exact significant differences were obtained between CD3+CD4+ vs CD19+ ($**P < 0.05$), CD19+ vs HLA-DR+ ($**P < 0.05$) and CD19+ vs CD56+ ($*P < 0.05$).

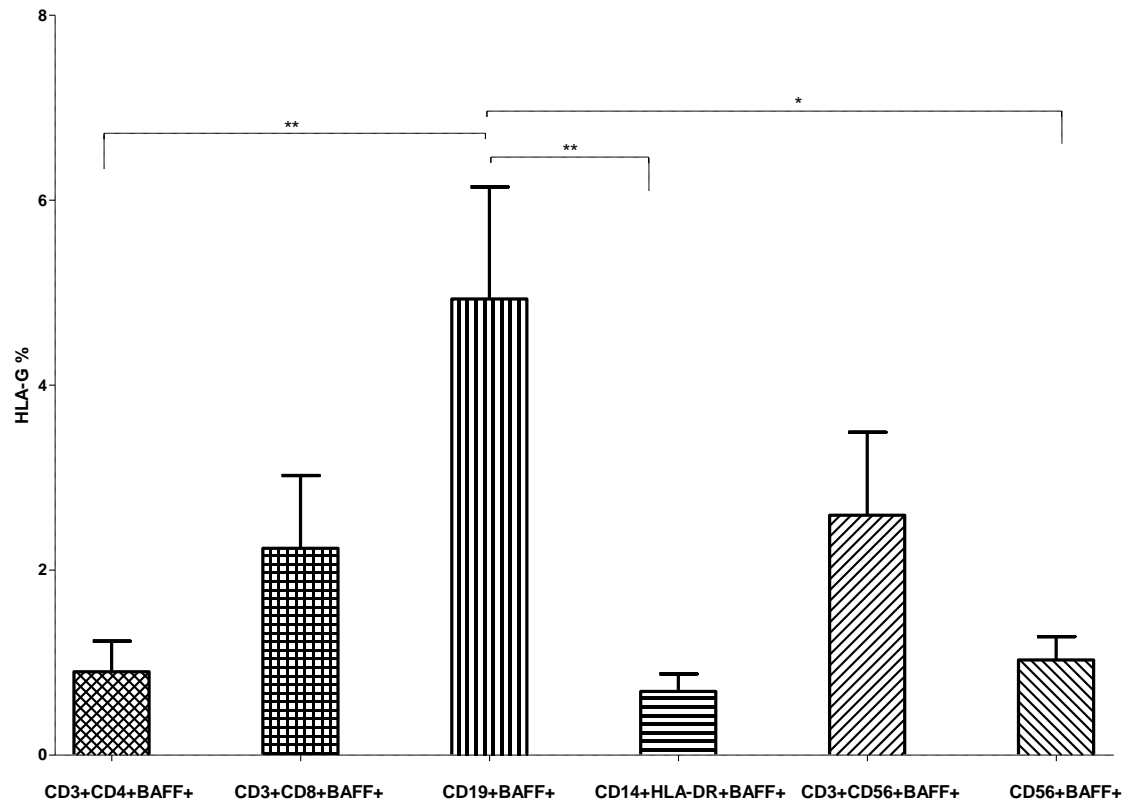


Fig. 6.17. The expression of HLA-G on BAFF + PBMCs following culture without CMV antigen in CMV - individuals. Results are expressed as mean % positive cells +/- SEM, n = 4, *P<0.05, **P<0.01.

6.18. Comparison of HLA-G expression in BAFF+ PBMCs in control and stimulated groups in CMV- subjects

For the purpose of illustrating the differences between HLA-G expression on stimulated and non-stimulated PBMCs, a comparison was made (Fig. 6.18) between the tested groups utilising a two tail Paired t test. Most of the examined cells populations showed statistically significant differences $P < 0.05$ [CD3+CD4+ ($P = 0.0221$), CD3+CD8+ ($P = 0.0401$), CD19+ ($P = 0.0329$), CD3+CD56+ ($P = 0.0048$) and CD56+ ($P = 0.0246$)], while there was no statistical difference in CD14+HLA-DR+ cells ($P = 0.0510$) although the P value was close to significance.

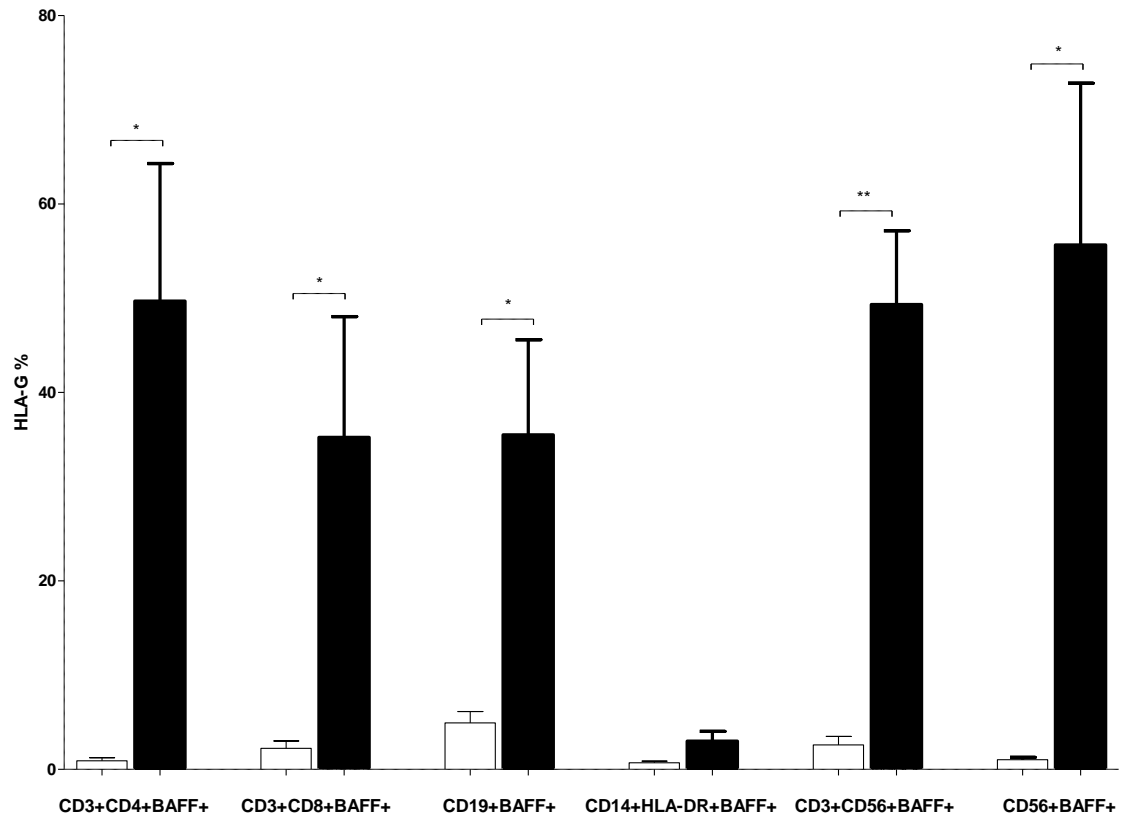


Fig. 6.18. Comparison of HLA-G expression on BAFF+ PBMC populations following culture with and without CMV antigen in CMV- individuals, empty columns (non-stimulated, n = 4) filled columns (stimulated, n = 4). Results are expressed as mean % HLA-G+ cells +/- SEM, *P<0.05, **P<0.01.

6.19. Expression of BAFF-R in cultured PBMCs induced with CMV antigen in CMV- subjects

In CMV- subjects, expression of the BAFF-R molecule was assessed in response to CMV antigen incubation for a week (Fig. 6.19). Generally, CD19+ cells were the highest group expressing the BAFF-R molecule (13.6%) and CD3+CD4+ lymphocytes were the lowest expresser of the antigen (1.6%). The rest of the cells expressed the protein in the following descending proportions: CD3+CD8+ (27.9%), CD3+CD56+ (21.0%), CD14+HLA-DR+ (17.5%) and CD56+ (14.3%). A comparison between the groups was performed with a one-way ANOVA (Tukey's Multiple Comparison Test) and there was a statistically significant difference with overall P=0.0001. The groups of cells that showed significant differences were CD3+CD4+BAFF-R+ vs CD19+BAFF-R+ (***P<0.01), CD3+CD8+BAFF-R+ vs

CD19+BAFF-R+ (**P<0.01), CD19+BAFF-R+ vs CD14+HLA-DR+BAFF-R+ (***P<0.01), CD19+BAFF-R+ vs CD3+CD56+BAFF-R+ (***P<0.01) and CD19+BAFF-R+ vs CD56+BAFF-R+ (***P<0.01).

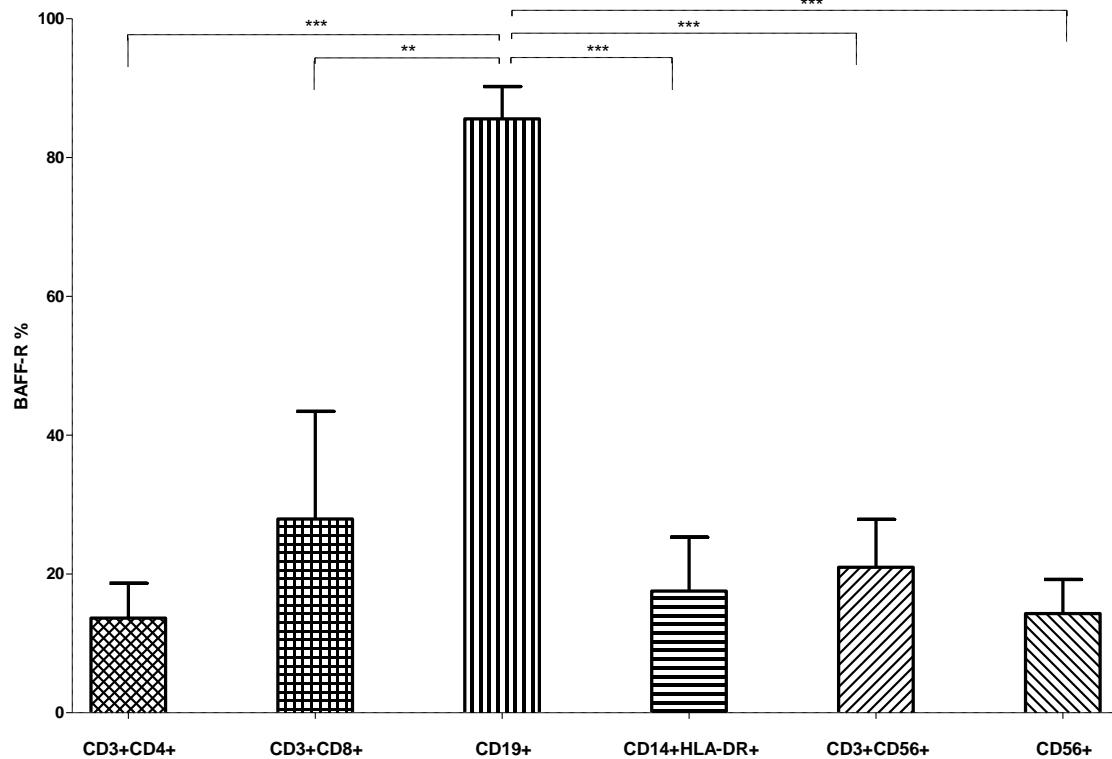


Fig. 6.19. The expression of BAFF-R on various PBMC populations following culture with CMV antigen in CMV- subjects. Results are expressed as mean +/- SEM, n = 4, **P<0.01, ***P<0.001.

6.20. Expression of BAFF-R in control PBMCs in CMV- subjects

Likewise, non-stimulated PBMC from CMV- subjects were assessed for the expression of the BAFF-R antigen after 7 days of culture and as demonstrated below (Fig. 6.20) a very high proportion of CD19+ cells expressed the molecule (94.76%), while the proportion of BAFF-R+ cells was lowest on CD14+HLA-DR+ (0.7%) cells. The other cells expressed the antigen in variable proportions: CD3+CD8+ (36.2%), CD3+CD4+ (13.9%), CD3+CD56+ (13.7%) and CD56+ cells (5.6%). When a one-way ANOVA (Tukey's Multiple Comparison Test) was applied, statistical difference

between the groups was noted with overall $P = 0.0001$. The following significant differences were observed between CD3+CD4+ vs CD19+ ($P < 0.001$), CD3+CD8+ vs CD19+ ($P < 0.001$), CD3+CD8+ vs CD14+HLA-DR+ ($P < 0.05$), CD19+ vs CD14+HLA-DR+ ($P < 0.001$), CD19+ vs CD3+CD56+ ($P < 0.001$) and CD19+ vs CD56+ cells ($P < 0.001$).

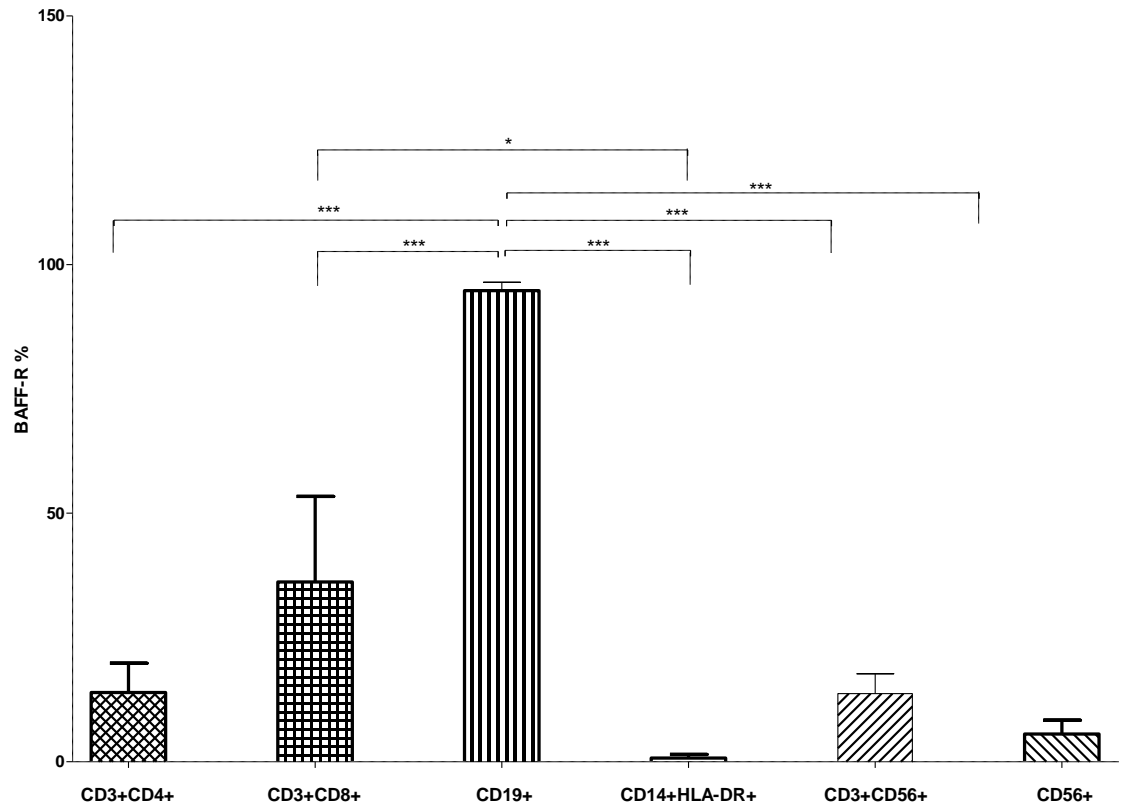


Fig. 6.20. The expression of BAFF-R on various PBMC populations following culture without CMV antigen in CMV - individuals. Results are expressed as mean % BAFF-R+ cells \pm SEM, $n = 4$, * $P < 0.05$, *** $P < 0.001$.

6.21. Comparison of BAFF-R expression in PBMCs cultured with and without CMV antigen in CMV- subjects

Stimulated and non-stimulated PBMCs from healthy CMV- individuals were compared after culture for a week (Fig. 6.21) using a two tailed Paired t test where there was no statistically significant difference between all the groups ($P > 0.05$),

although, the P values for CD19+ cells ($P=0.0619$) and CD56+ lymphocytes ($P=0.0668$) were close to significant levels. There were some patterns of upregulation in BAFF-R expression following culture with CMV antigens particularly in CD14+HLA-DR+ cells, CD3+CD56+ lymphocytes and CD56+ cells. However, lower proportions of BAFF-R+ cells were found in the induced cells, especially CD3+CD8+ and CD19+ lymphocytes.

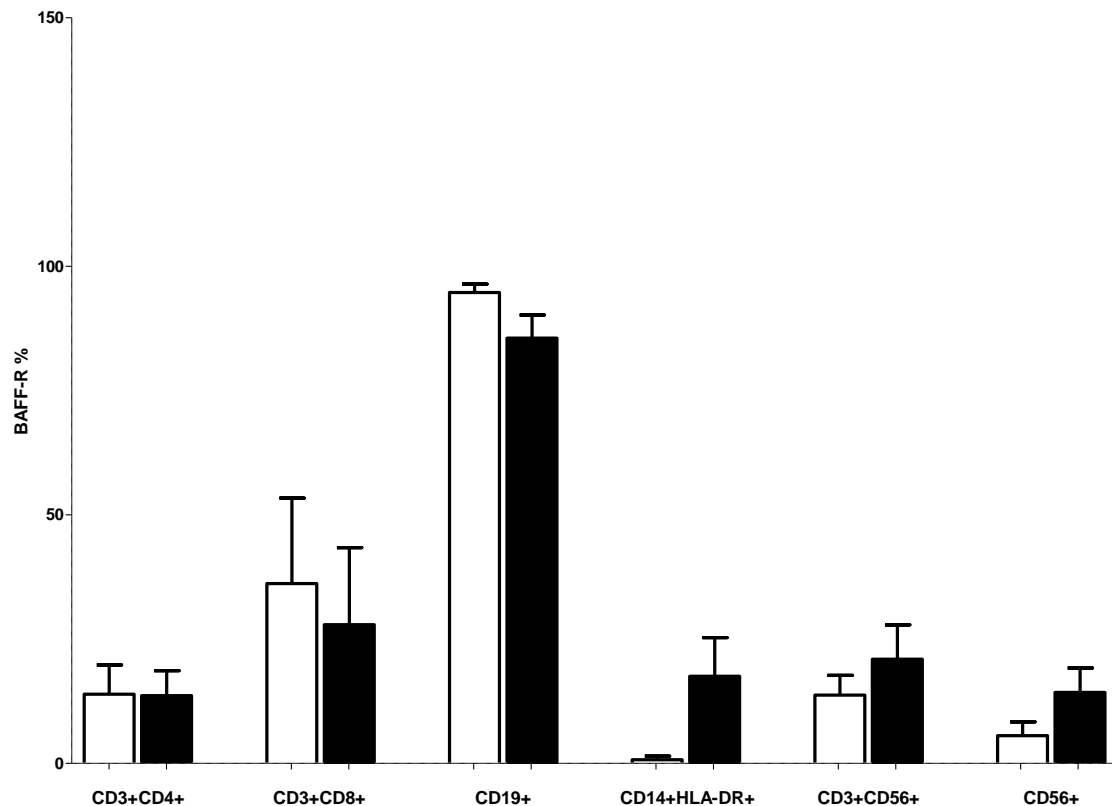


Fig. 6.21. Comparison of BAFF-R expression on various PBMC populations following culture with and without CMV antigen in CMV- subjects. Empty columns (non-stimulated, $n = 4$), filled columns (stimulated, $n = 4$). Results are expressed as mean \pm SEM, no statistically significant differences were noted ($P>0.05$).

6.22. Expression of HLA-G on BAFF-R+ PBMCs cultured with CMV antigen in CMV- subjects

HLA-G expression was evaluated on those PBMCs that expressed the BAFF-R as previously described. In (Fig. 6.22), CD3+CD56+ lymphocytes were the highest expresser of HLA-G (62.8%) among the tested groups, while the proportions of

HLA-G were lowest in CD19+ cells (7.2%). There were close proportions of HLA-G expressing cells in CD3+ lymphocytes (10.8%) and CD3+CD8+ cells (11.4%) and the percentage was slightly higher in CD3+CD4+ cells (16.3%) and CD56+ lymphocytes (23.5%). After testing the differences between the groups with a one-way ANOVA (Tukey's Multiple Comparison Test), there were statistically significant differences among them with overall $P=0.0005$. The significant differences were recorded between CD3+CD4+ vs CD3+CD56+ ($P<0.05$), CD3+CD8+ vs CD3+CD56+ ($P<0.01$) and CD19+ vs CD3+CD56+ cells ($P<0.01$).

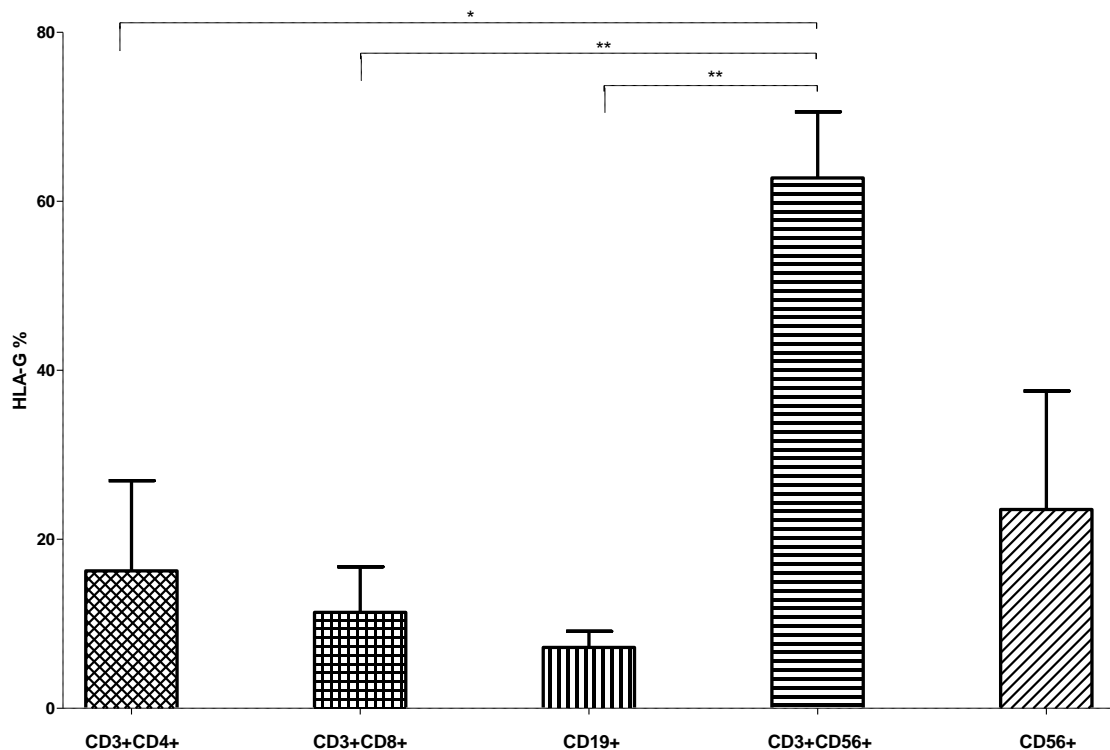


Fig. 6.22. The expression of HLA-G on BAFF-R + PBMC populations following culture with CMV antigen in CMV- subjects. Results are expressed as mean \pm SEM, $n = 4$, * $P<0.05$, ** $P<0.01$.

6.23. Expression of HLA-G on BAFF-R+ PBMCs in control CMV-subjects

Similarly, in non-stimulated CMV- individuals, the HLA-G expression was measured with flow cytometry as shown in (Fig. 6.23). The proportion of HLA-G+ cells was highest in BAFF-R+ CD3+CD56+ cells (2.4%), whereas the remaining cell

populations expressed the following descending proportions: CD19+ (1.3%), CD56+ (1.0%), CD3+CD4+ (0.94%) and CD3+CD8+ (0.86%). Upon testing the differences among the groups utilizing a one-way AONVA (Tukey's Multiple Comparison Test), no statistically significant difference was noted ($P=0.4219$).

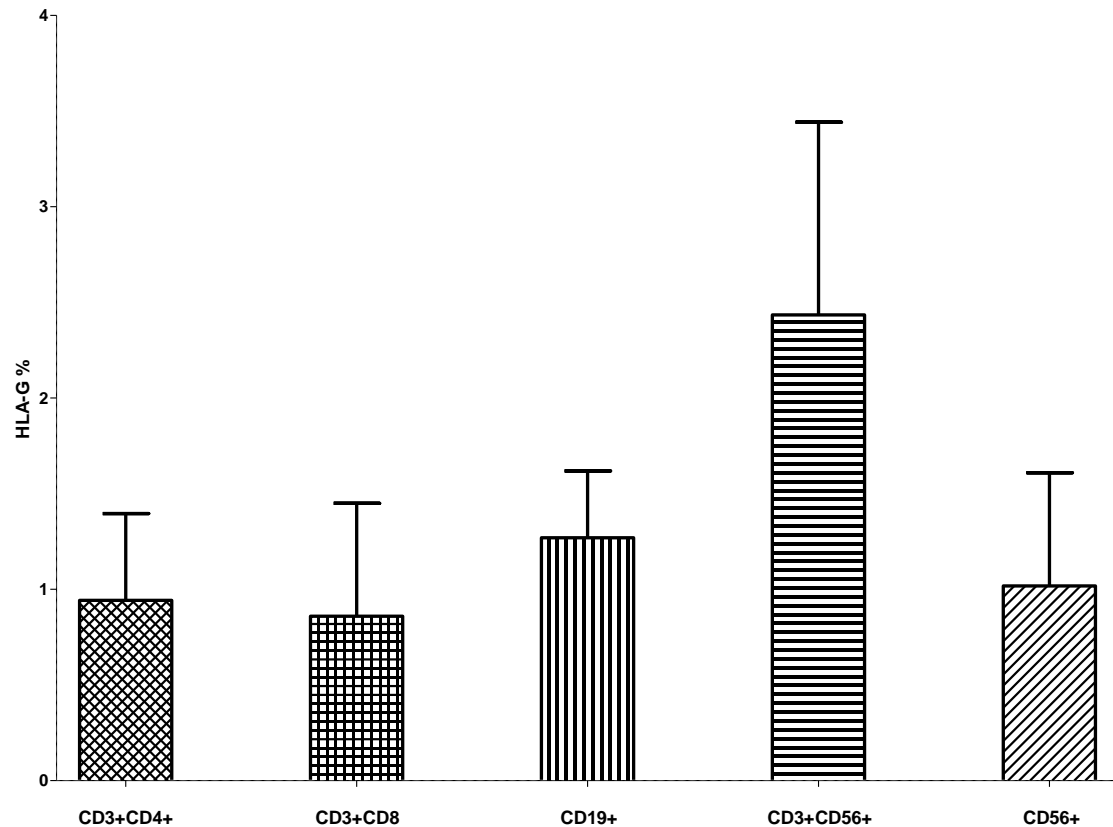


Fig. 6.23. The expression of HLA-G on BAFF-R+ PBMC populations following culture with CMV antigen in CMV- subjects. Results are expressed as mean \pm SEM, $n = 4$, no statistically significant difference was noted among the groups $P>0.05$.

6.24. Comparison of HLA-G expression on BAFF-R+ PBMCs in control and induced cells in CMV- subjects

HLA-G expression on BAFF-R+ PBMCs was compared between the examined groups that were cultured with and without CMV antigens (Fig. 6.24). A two-tailed Paired t test was applied to test the differences among them and some subpopulations noted statistically significant differences ($P<0.05$), particularly in CD19+ lymphocytes ($P=0.0430$), CD3+CD56+ cells ($P=0.0023$) and CD3+CD8+ cells ($P=0.0117$), unlike

the remaining subsets [CD3+CD4+ ($P=0.1256$), and CD56+ ($P= 0.1082$)] where no significant differences were recorded between them ($P>0.05$).

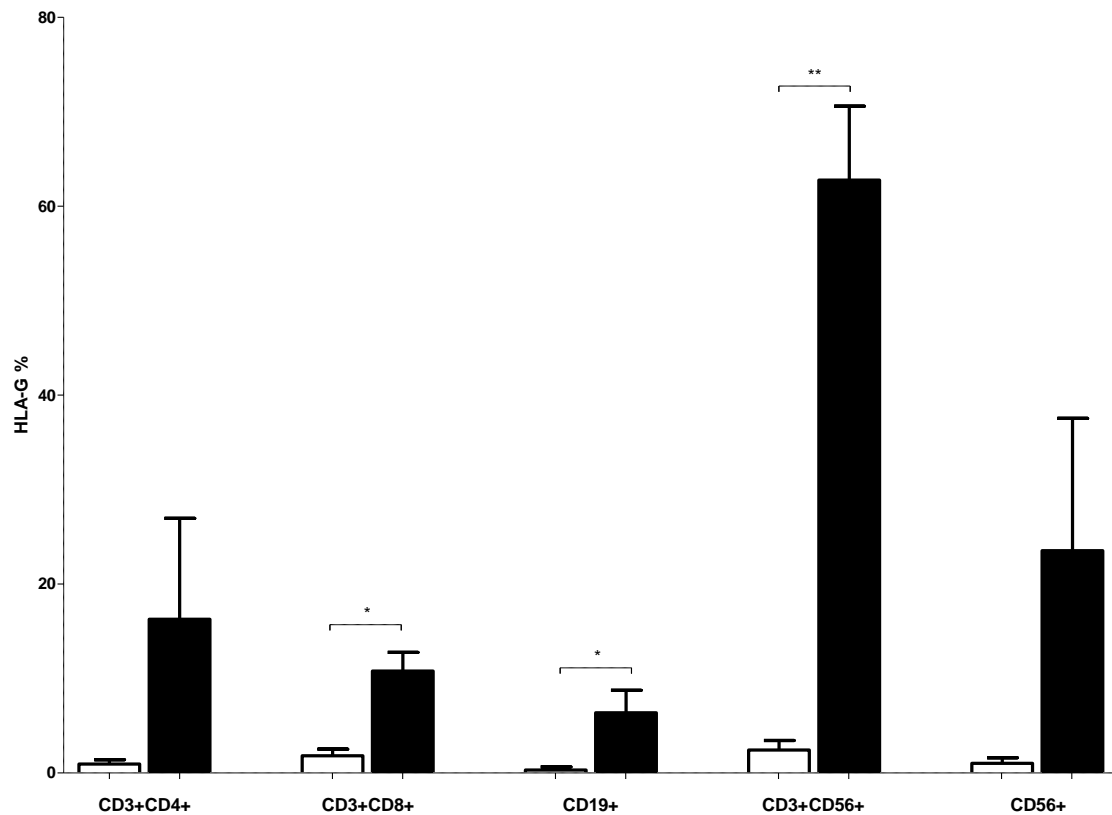


Fig. 6.24. The expression of HLA-G on BAFF-R+ PBMC populations following culture with or without CMV antigen in CMV- subjects. Empty columns (non-stimulated, $n = 4$), filled columns (stimulated, $n = 4$). Results are expressed as mean \pm SEM, * $P<0.05$, ** $P<0.01$.

6.25. Comparison of BAFF expression in PBMCs cultured with CMV antigen in CMV- and CMV+ subjects

In order to assess the expression of BAFF antigen by CMV stimulated PBMCs following culture for a week in CMV- and CMV+ healthy subjects, a comparison was made (Fig. 6.25) with a two tail Mann Whitney test. The test disclosed no statistically significant differences between all the examined pairs of cells ($P>0.05$), though nearly all the CMV+ populations were displaying higher proportions of BAFF molecule than the CMV- ones except for CD3+CD56+ lymphocytes which expressed the opposite.

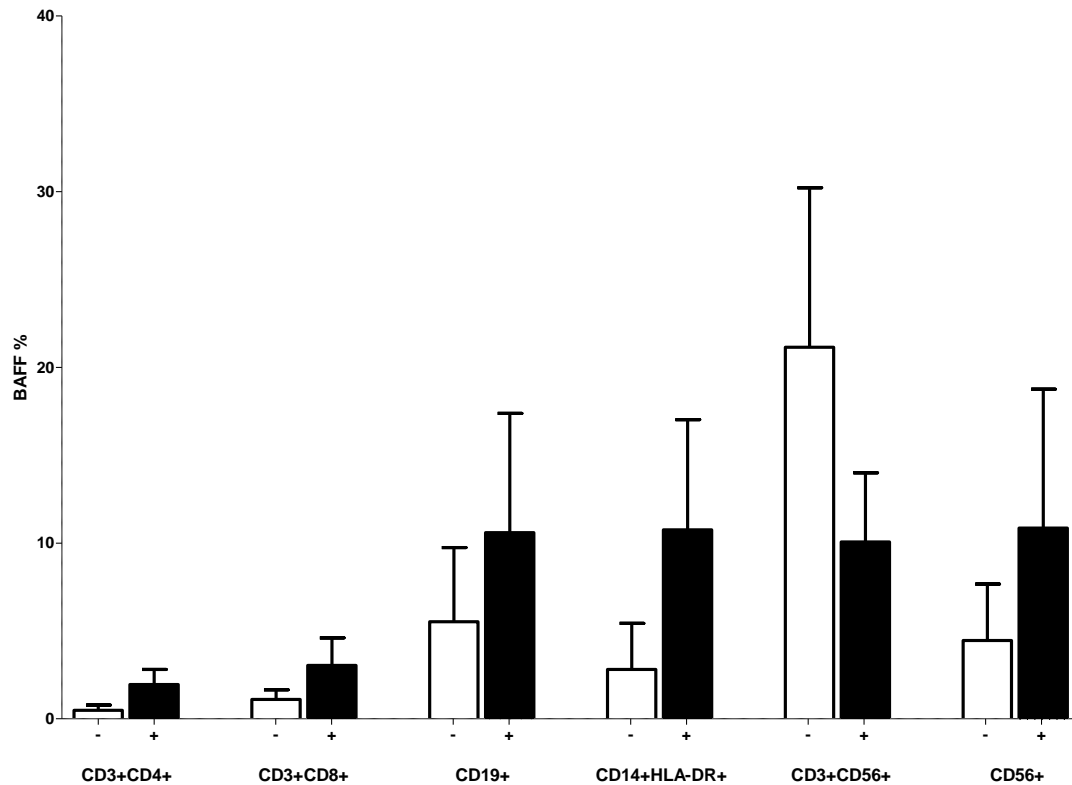


Fig. 6.25. Comparison of BAFF expression on PBMC populations following culture with CMV antigens in CMV- and CMV+ individuals, empty columns (CMV -, n = 4) filled columns (CMV +, n = 5). Results are expressed as mean \pm SEM, no statistically significant differences were noted ($P > 0.05$).

6.26. Comparison of HLA-G expression on BAFF+ PBMCs induced with CMV antigen in CMV- and CMV+ subjects

The expression of HLA-G on PBMCs that expressed the BAFF antigen was compared between CMV- and CMV+ subjects after culture with CMV antigens (Fig. 6.26). The groups were compared with a two tailed Mann Whitney test and there were no statistically significant differences $P > 0.05$ between all the populations. Interestingly, the proportions of HLA-G+ cells displayed by the CMV- subjects were higher than CMV+ individuals in nearly all the subpopulations apart from CD14+HLA-DR+ cells which was slightly higher in CMV+ subjects.

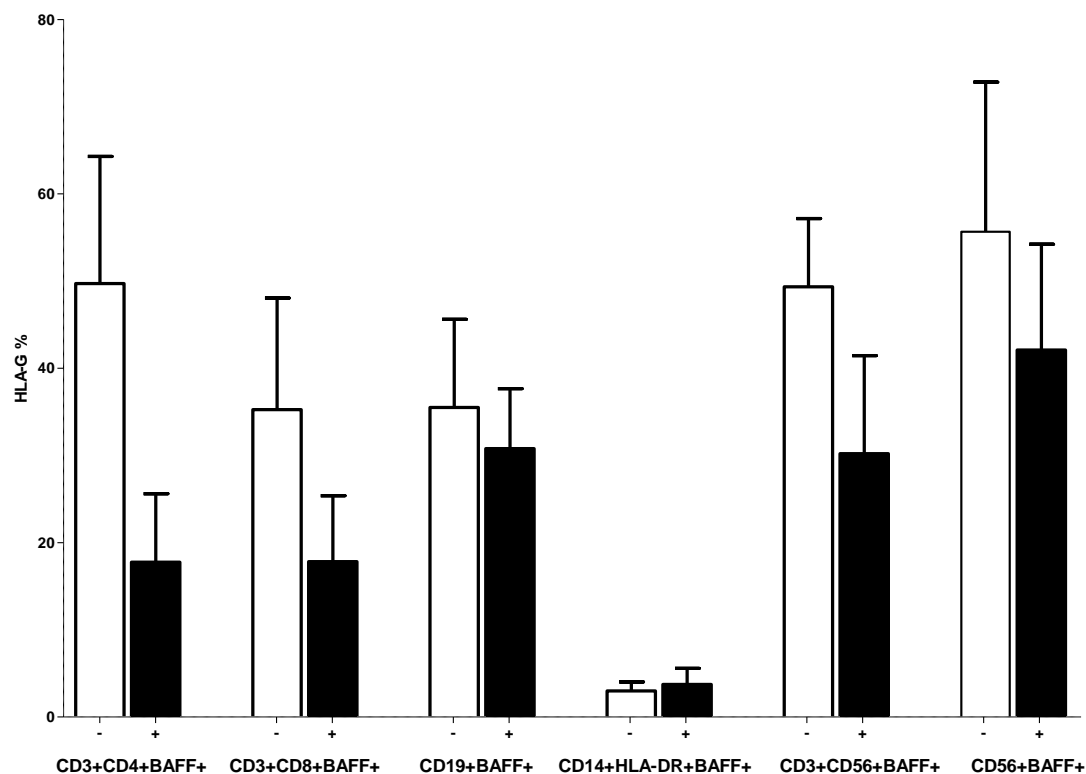


Fig. 6.26. Comparison of HLA-G expression on BAFF + PBMCs following culture with CMV antigens in CMV - and CMV + individuals, empty columns (CMV -, n = 4) filled columns (CMV +, n = 5). Results are expressed as mean \pm SEM, no statistically significant differences were noted ($P > 0.05$).

6.27. Comparison of BAFF-R+ PBMCs cultured with CMV antigen in CMV- and CMV+ subjects

In the same way, BAFF-R expression in PBMCs from CMV- and CMV+ individuals following culture with CMV antigens (Fig. 6.27) was compared to elucidate any differences between the groups. After applying a two tailed Mann Whitney test, there were no statistically significant differences between the entire pairs ($P > 0.05$). The proportions of BAFF-R+ cells in CMV+ subjects were relatively higher in CD3+CD4+, CD3+CD8+ and CD56+ lymphocytes while in the other groups (CD19+ cells, CD14+HLA-DR+ cells and CD3+CD56+ lymphocytes) the proportions of BAFF-R expressing cells was slightly higher in CMV- subjects.

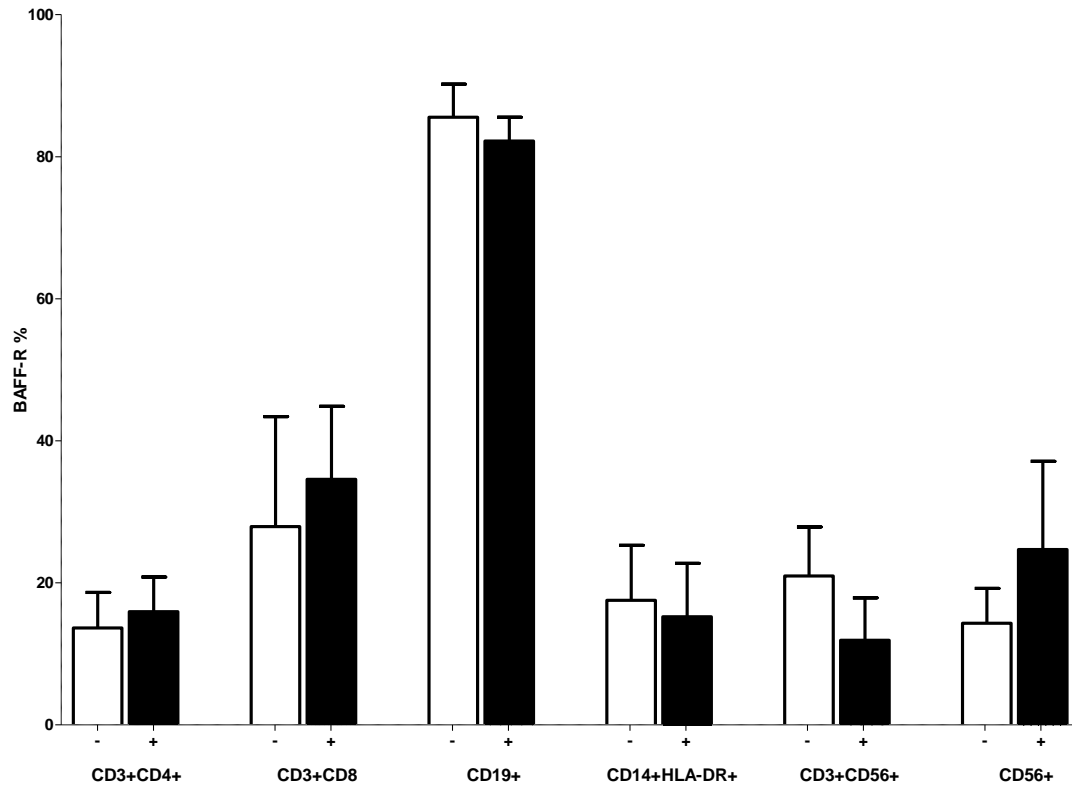


Fig. 6.27. Comparison of BAFF-R expression on PBMC populations following culture with CMV antigens in CMV- and CMV+ individuals, empty columns (CMV-, n = 4) filled columns (CMV+, n = 5). Results are expressed as mean \pm SEM, no statistically significant differences were noted $P > 0.05$.

6.28. Comparison of HLA-G expression on BAFF-R+ PBMCs induced with CMV antigen in CMV- and CMV+ donors

Similar to the approach above, HLA-G was compared in PBMCs expressing BAFF-R in CMV- and CMV+ subjects after CMV induction (Fig. 6.28). Upon applying a two tail Mann Whitney test, no statistically significant differences ($P > 0.05$) were recorded between all the examined populations. However, the proportion of HLA-G expressing BAFF-R+ cells was slightly higher in CMV- individuals, particularly in CD3+CD4+, CD3+CD8+, CD19+, CD3+CD56+ and CD56+ cells.

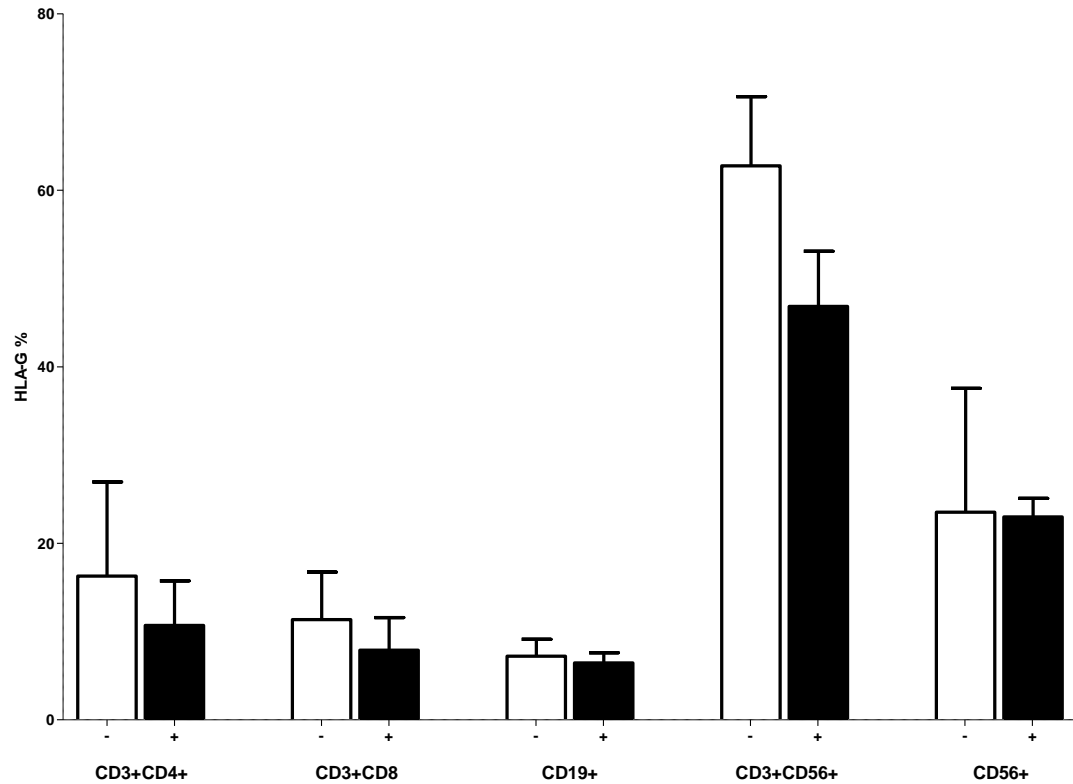


Fig. 6.28. Comparison of HLA-G expression on BAFF-R PBMCs following culture with CMV antigens in CMV- and CMV+ individuals, empty columns (CMV-, n = 4) filled columns (CMV+, n = 5). Results are expressed as mean \pm SEM, no statistically significant differences were noted between the groups ($P > 0.05$).

6.29. Expression of BAFF on PBMCs culture without CMV antigen

The expression of BAFF protein in control cultured PBMCs without CMV antigens from both CMV- and CMV+ healthy subjects was evaluated (Fig. 6.29) and the highest proportion of BAFF+ cells was noted in CD3+CD56+ cells (9.98%) while very low percentages were recorded in CD3+CD4+ cells and CD3+CD8+ lymphocytes (0.28% and 0.58%). The remaining groups have expressed the following proportions; CD14+HLA-DR+= 3.79%, CD19+= 2.89% and CD56+= 2.22%.

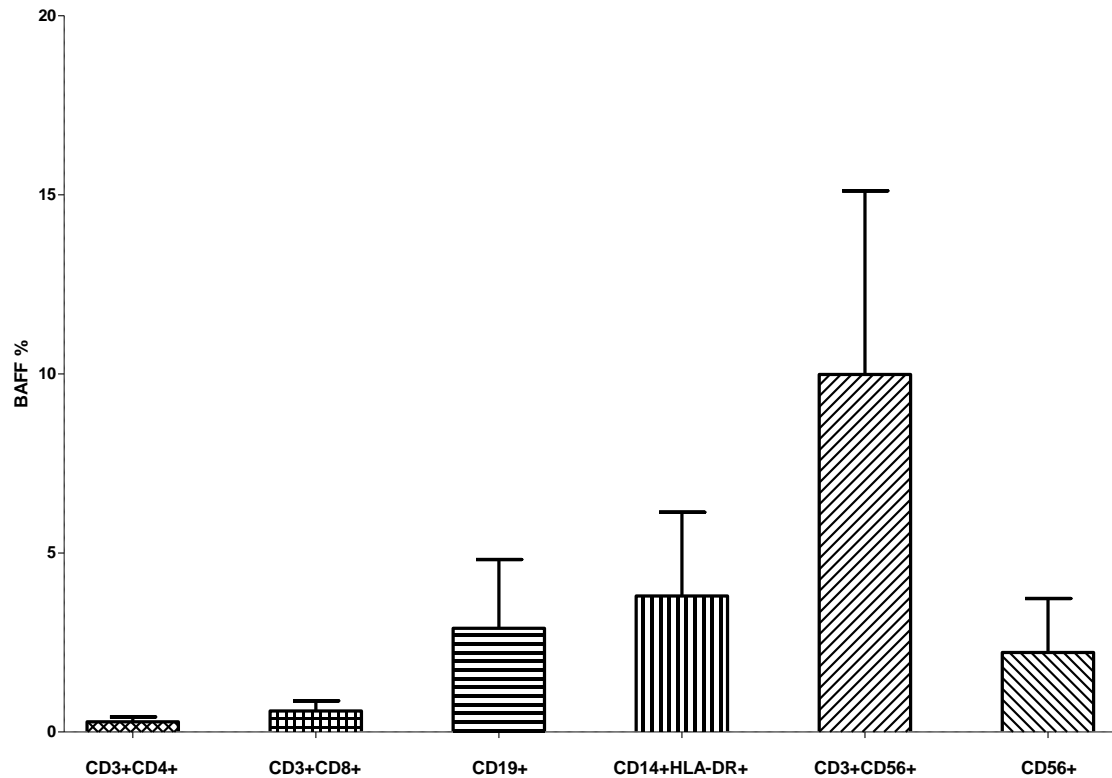


Fig. 6.29. Expression of BAFF in control PBMCs following culture without CMV antigens. Results are expressed as mean \pm SEM, n = 9.

6.30. Expression of BAFF on PBMCs culture with CMV antigen

Expression of BAFF molecule was assessed on PBMCs from CMV- and CMV+ healthy subjects cultured with CMV antigens for a week (Fig. 3.30) and highest proportion of this receptor was displayed by CD3+CD56+ cells (14.99%) followed by CD19+ cells (8.34%) then CD56+ cells 8.0%, CD14+HLA-DR+ cells 7.22%, CD3+CD8+ lymphocytes 2.18% and CD3+CD4+ cells 1.3%.

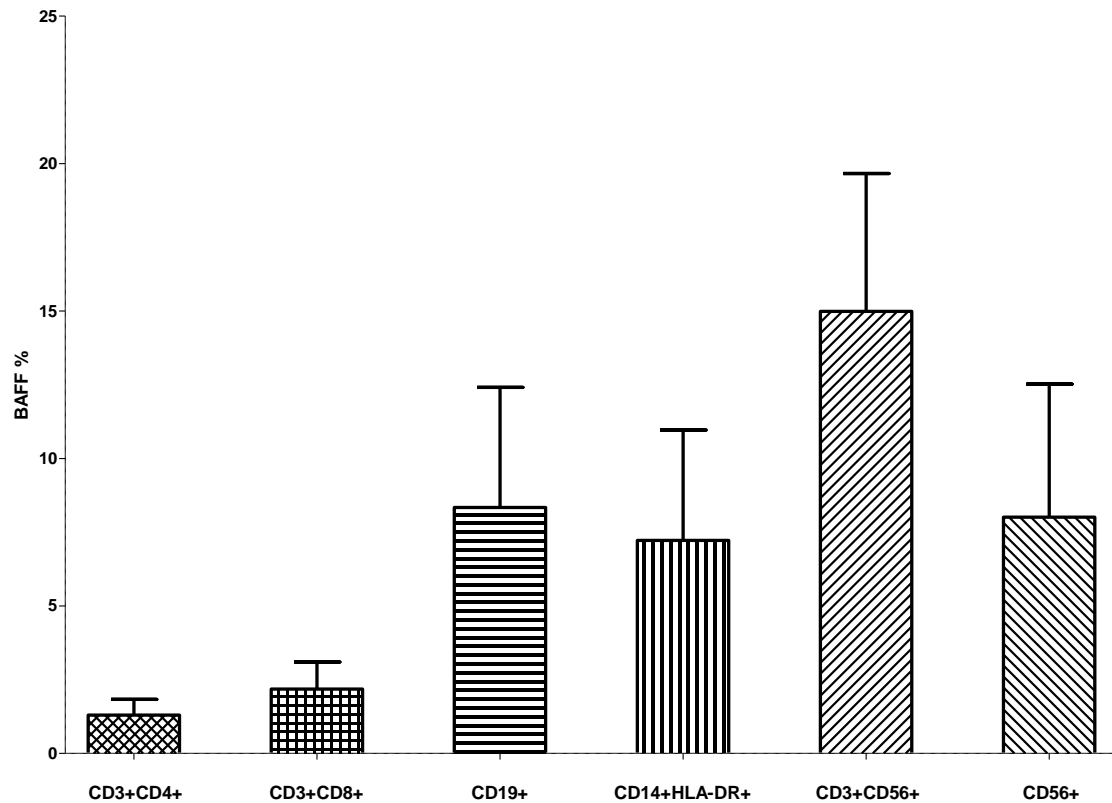


Fig. 6.30. Expression of BAFF on PBMCs following culture with CMV antigens. Results are expressed as mean \pm SEM, n = 9.

6.31. Comparison of BAFF expression on PBMCs culture with and without CMV antigen

Data gathered from induced and non-induced PBMCs from the two graphs above were plotted (Fig. 6.31) to illustrate the changes after CMV induction, a comparison was made using Paired t test (two tails). No statistically significant differences were recorded between all the compared subpopulations $P > 0.05$.

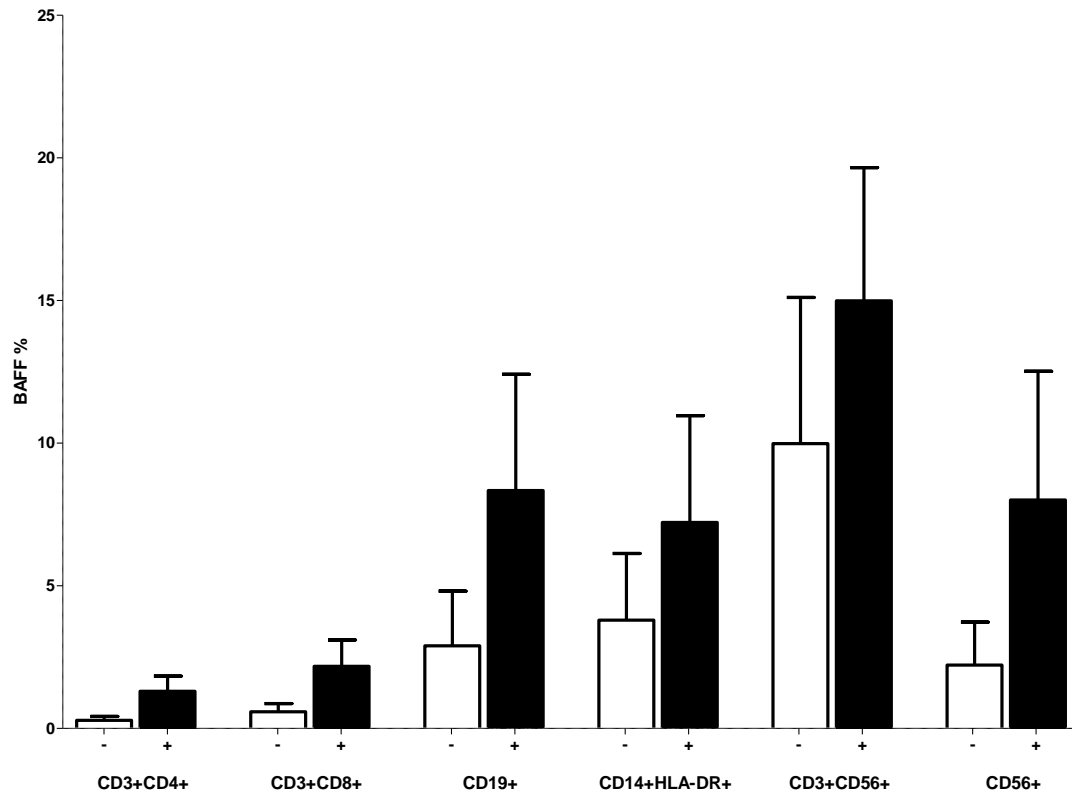


Fig. 6.31. Comparison of BAFF expression on PBMCs following culture with and without CMV antigens. Results are expressed as mean \pm SEM, $n = 9$, Empty columns (control) and filled columns (stimulated). $P > 0.05$.

6.32. Expression of HLA-G on BAFF+ PBMCs culture without CMV antigen

The HLA-G expression was measured on PBMCs displayed BAFF+ molecule after culture for a week without CMV antigen (Fig. 6.32) and there were relatively low proportion of HLA-G expressing BAFF+ cells with highest percentage being displayed by CD19+ cells 3.26%, while the remainder cell populations had the following proportions; CD3+CD56+= 1.49%, CD3+CD8+= 1.38%, CD56+= 0.61%, CD3+CD4+= 0.59% and CD14+HLA-DR+= 0.32%.

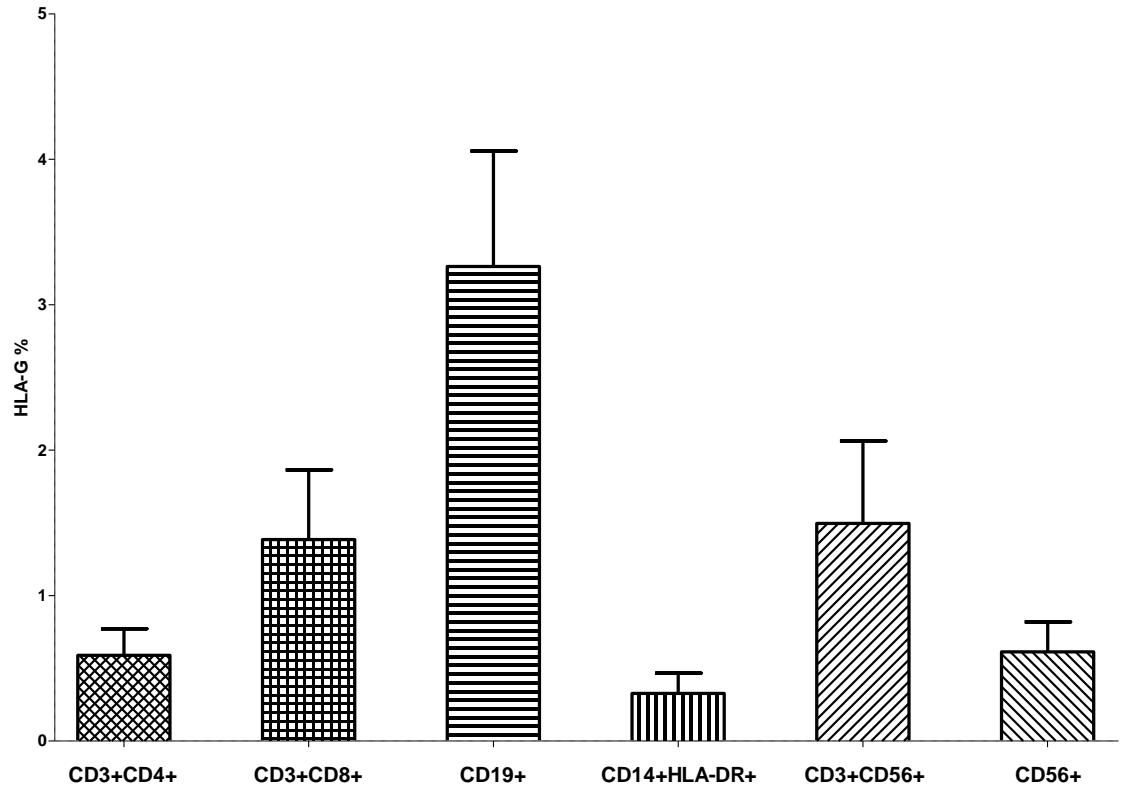


Fig. 6.32. Expression HLA-G on BAFF+ PBMCs following culture without CMV antigens. Results are expressed as mean \pm SEM, n = 9.

6.33. Expression of HLA-G on BAFF+ PBMCs culture with CMV antigen

Similarly, HLA-G expression by BAFF+ PBMCs following CMV induction for a week was measured (Fig. 6.33) and there was marked upregulation of HLA-G on BAFF+ cells with close proportions recorded as follow; CD56+=48.12%, CD3+CD56+=38.7%, CD19+=32.87%, CD3+CD4+=31.96%, CD3+CD8+=25.57% and CD14+HLA-DR+=3.41%.

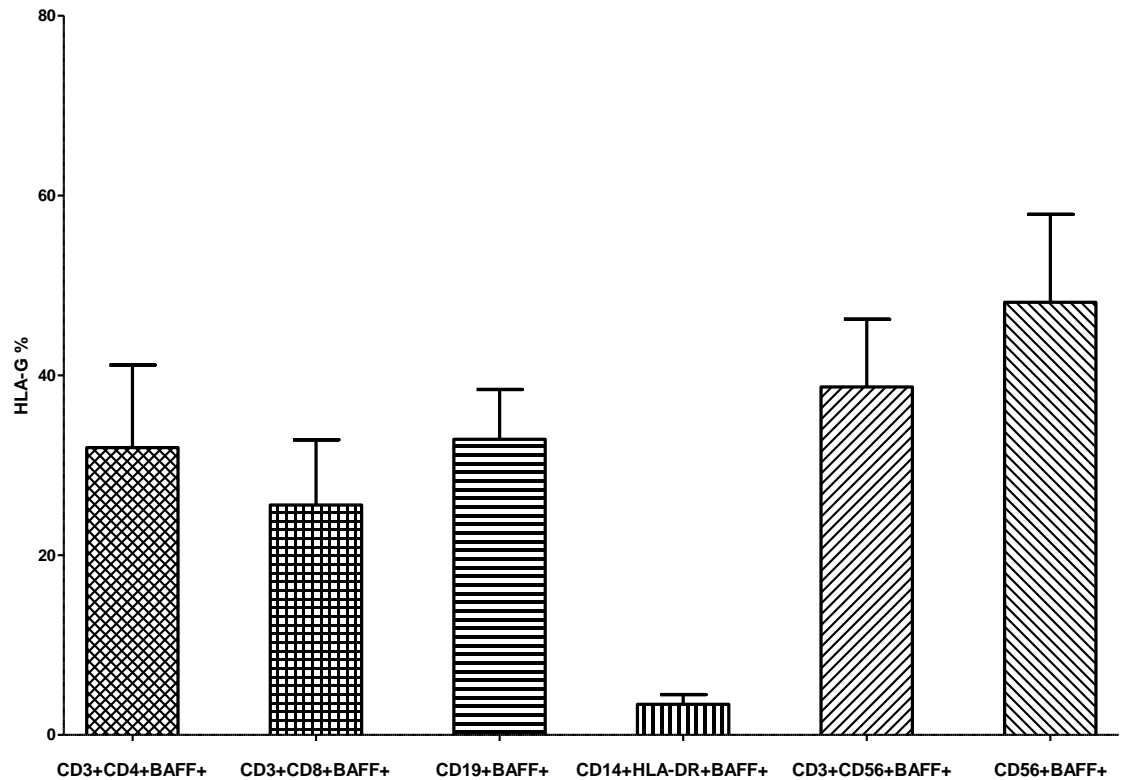


Fig. 6.33. Expression HLA-G on BAFF+ PBMCs following culture with CMV antigens. Results are expressed as mean \pm SEM, n = 9.

6.34. Comparison of HLA-G expression on BAFF+ PBMCs culture with and without CMV antigen

Induced and non-induced PBMCs were plotted in the graph below to demonstrate the changes in HLA-G expression in BAFF+ cells following culture (Fig. 6.34). Upon performing Paired t test (two tails), significant differences were obtained in the all cell groups (CD3+CD4+ $P = 0.0086$, CD3+CD8+ $P = 0.0086$, CD19+ $P = 0.0008$, CD14+HLA-DR+ $P = 0.0222$, CD3+CD56+ $P = 0.0010$ and CD56+ $P = 0.0012$).

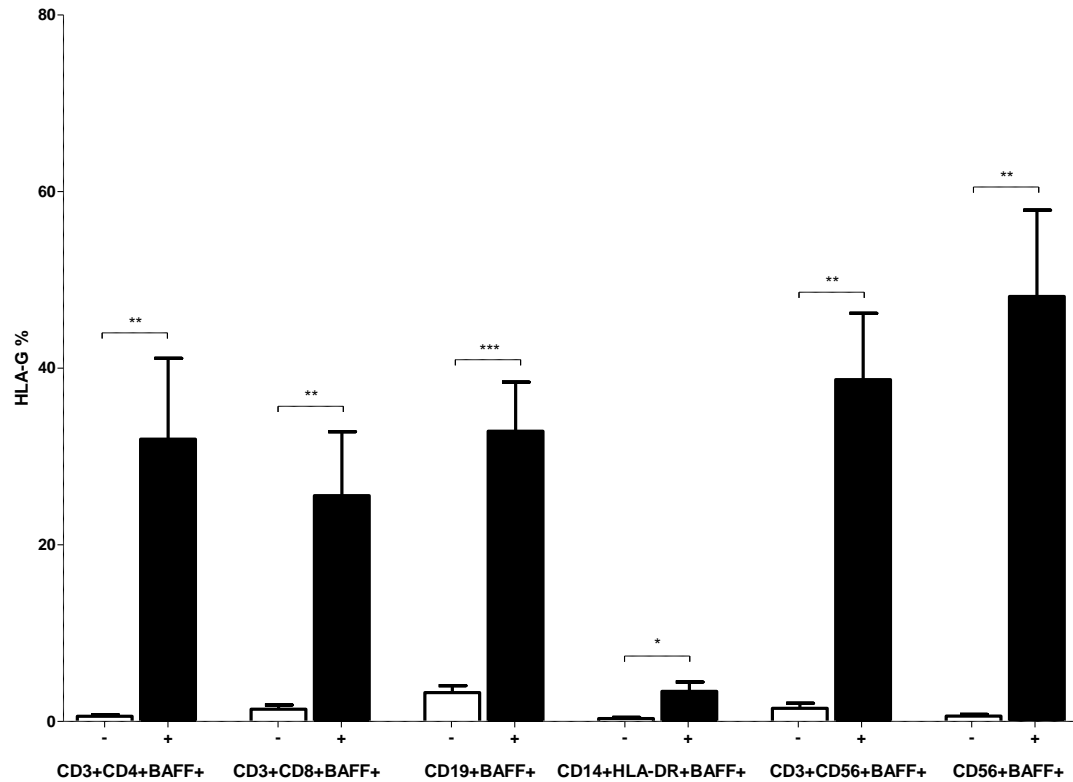


Fig. 6.34. Comparison of HLA-G expression on BAFF+ PBMCs following culture with and without CMV antigens. Results are expressed as mean \pm SEM, $n = 9$. Empty columns (non-stimulated) and filled columns (stimulated). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

6.35. Expression of BAFF-R on PBMCs culture without CMV antigen

Similar approach was followed with BAFF-R, the expression of this receptor on PBMCs cultured without CMV antigen for a week was measured (Fig. 6.35). Highest proportion was noted in CD19+ cells 95.82%, whereas, the other groups of cells displayed the following percentages; CD3+CD8+= 38.24%, CD3+CD4+= 10.77%, CD56+= 9.2%, CD14+HLA-DR+= 8.43% and CD3+CD56+= 6.94%.

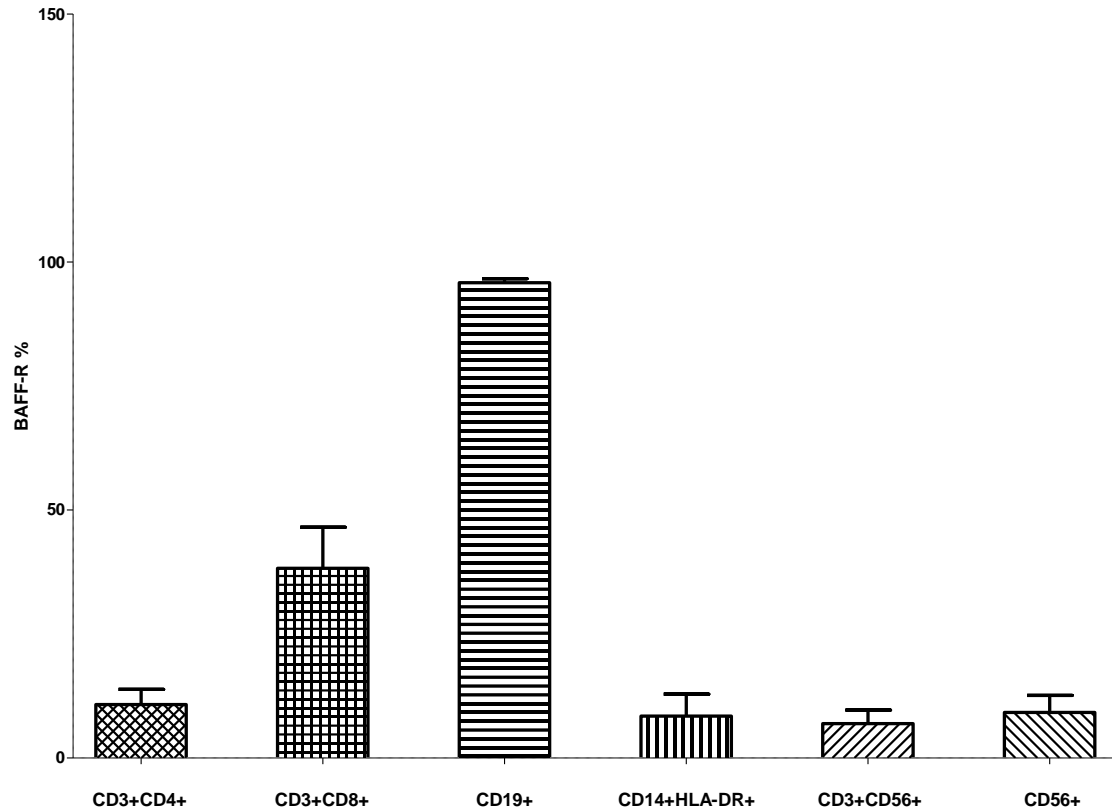


Fig. 6.35. Expression on BAFF-R on PBMCs following culture without CMV antigens. Results are expressed as mean \pm SEM, n = 9.

6.36. Expression of BAFF-R on PBMCs culture with CMV antigen

Likewise, BAFF-R expression was recorded on PBMCs cultured with CMV antigen for a week (Fig. 6.36) and elevated proportion was observed on CD19+ cells 83.70%. The other cell population recorded the following percentages; CD3+CD8+= 31.61%, CD56+= 20.06%, CD14+HLA-DR+= 16.25%, CD3+CD56+= 15.94% and CD3+CD4+= 14.91%.

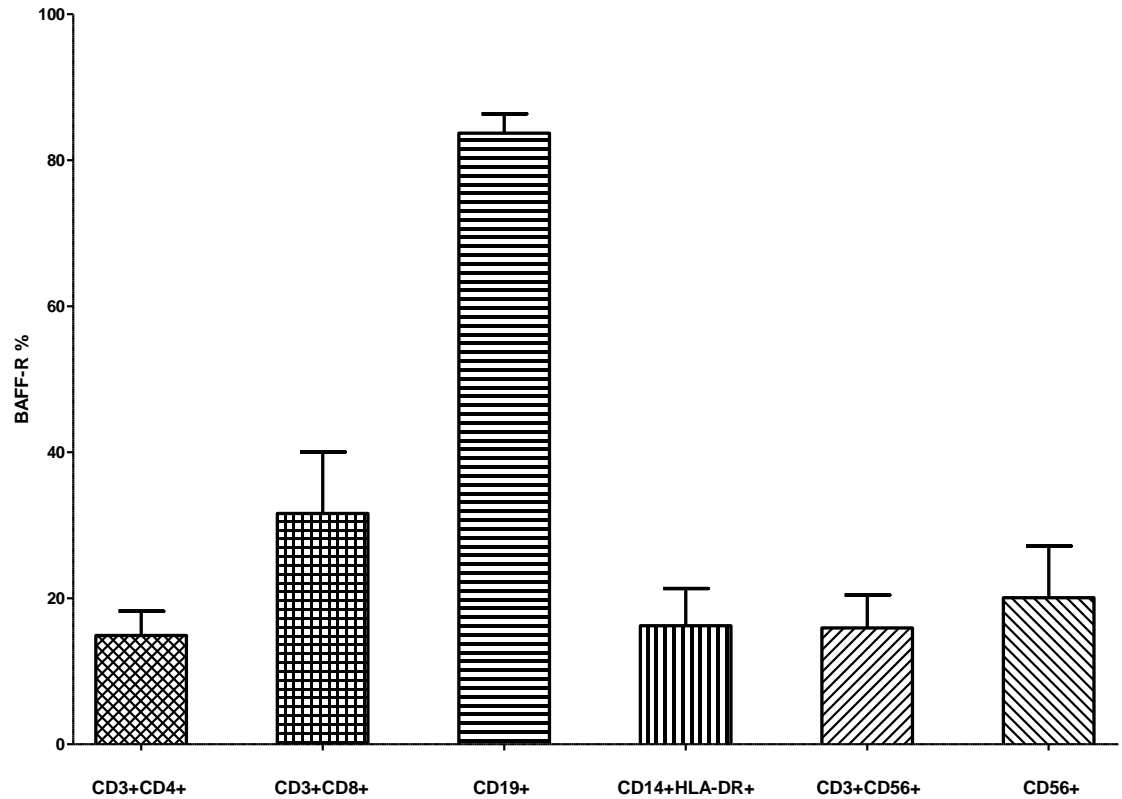


Fig. 6.36. Expression on BAFF-R on PBMCs following culture with CMV antigens. Results are expressed as mean \pm SEM, n = 9.

6.37. Comparison of BAFF-R expression on PBMCs culture with and without CMV antigen

The changes in BAFF-R expression were assessed between CMV induced and non-induced cells as depicted in the graph (Fig. 6.37), the differences between the groups were tested with Paired t test (two tails) and there were significant differences only in CD19+ cells ($P = 0.0009$) and CD56+ cells ($P = 0.0273$). No statistically significant differences were obtained between the remaining cells $P > 0.05$.

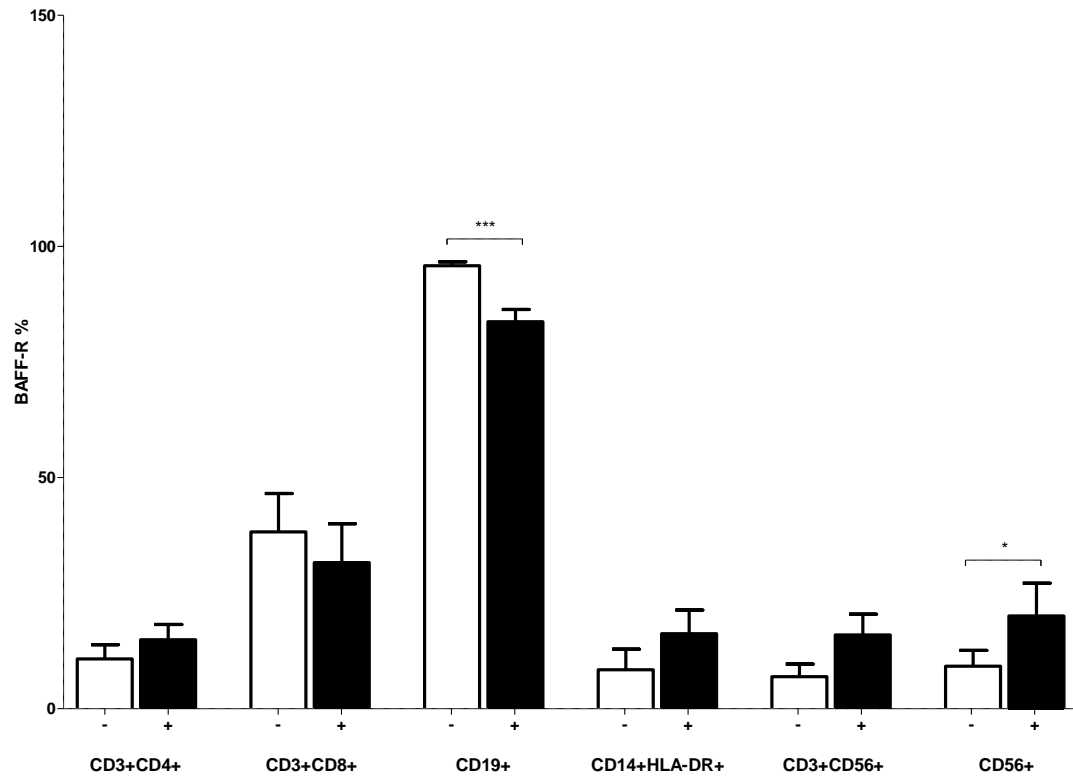


Fig. 6.37. Comparison of BAFF-R expression on PBMCs following culture with and without CMV antigens. Results are expressed as mean \pm SEM, $n = 9$. Empty columns (non-stimulated) and filled columns (stimulated). * $P < 0.05$ and *** $P < 0.001$

6.38. Expression of HLA-G on BAFF-R+ PBMCs culture without CMV antigen

In the same way, HLA-G expression on BAFF-R+ cells was measured following culture without CMV antigens (Fig. 6.38) and overall low proportions was observed in all the tested populations CD3+CD56+= 2.12%, CD19+= 1.1%, CD3+CD4+= 0.94%, CD56+= 0.85% and CD3+CD8+= 0.69%.

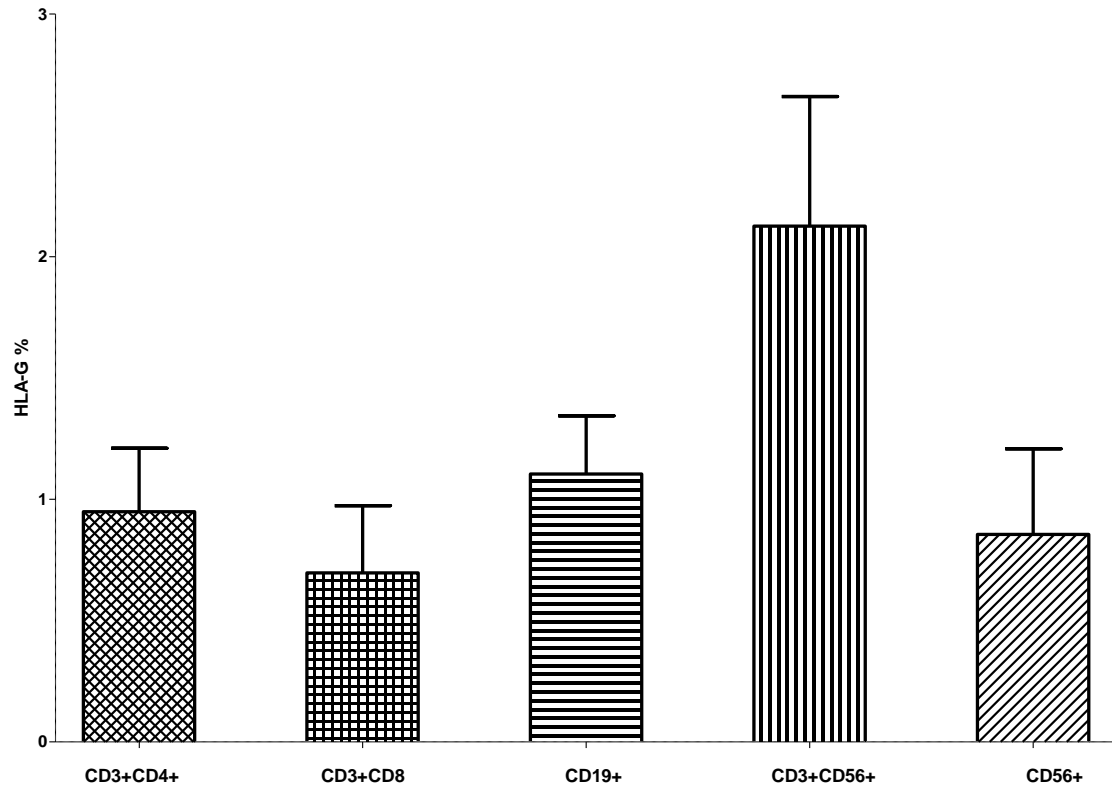


Fig. 6.38. Expression of HLA-G on BAFF-R+ PBMCs following culture without CMV antigens. Results are expressed as mean \pm SEM, n = 9.

6.39. Expression of HLA-G on BAFF-R+ PBMCs culture with CMV antigen

Following CMV induction for a week, HLA-G expression on BAFF-R+ PBMCs was examined and there were elevated proportions noted on some of the tested groups (CD3+CD56+= 53.91%, CD56+= 23.22%, CD3+CD4+= 13.18%, CD3+CD8+= 9.43% and CD19+= 6.78%) (Fig. 6.39).

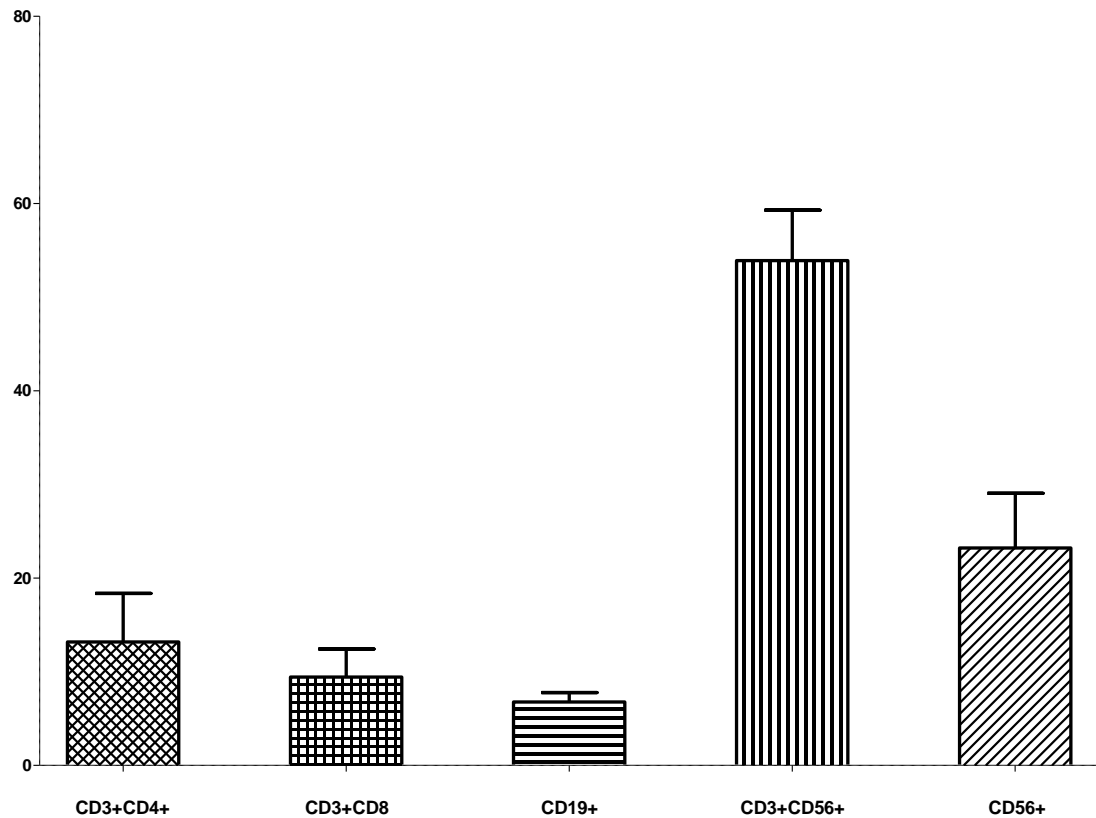


Fig. 6.39. Expression of HLA-G on BAFF-R+ PBMCs following culture with CMV antigens. Results are expressed as mean +/- SEM, n = 9.

6.40. Comparison of HLA-G expression on BAFF-R+ PBMCs culture with and without CMV antigen

The changes in HLA-G expression between CMV induced and non-induced were measured in BAFF-R+ cells (Fig. 6.40) and after applying Paired t test (two tails), statistically significant differences were obtained in all cell groups (CD3+CD4+ $P=0.0455$, CD3+CD8+ $P=0.0160$, CD19+ $P=0.0002$, CD3+CD56+ $P<0.0001$ and CD56+ $P=0.0058$).

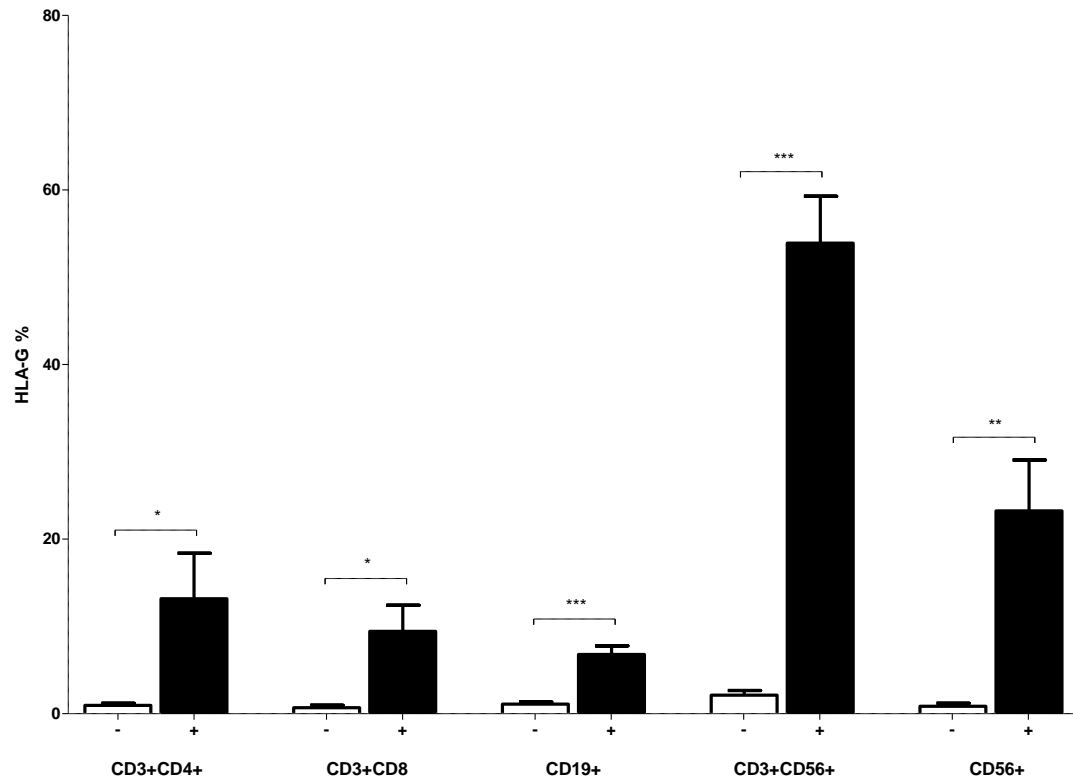


Fig. 6.40. comparison of HLA-G expression on BAFF-R+ PBMCs following culture with and without CMV antigens. Results are expressed as mean \pm SEM, $n = 9$. Empty columns (non-stimulated) and filled columns (stimulated). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

6.41. sBAFF levels in the plasma from healthy subjects and culture supernatants

An ELISA test was performed to assess the levels of BAFF antigen in the plasma from healthy CMV- and CMV+ subjects (Table 6.3). The assay was performed on frozen samples extracted at the time of PBMC separation. The groups were compared to assess the difference in BAFF levels in relation to CMV status and after applying a two tailed Mann Whitney test, there was no statistically significant difference between the groups ($P = 0.6417$). Likewise, culture supernatants from cells stimulated with or without CMV antigens for a week were harvested and tested with sBAFF ELISA, the results were calculated and the values obtained were compared with a two tailed Paired t-test and no statistically significant differences was noted ($P = 0.0610$).

Serum	CMV-	CMV+	P value
n =	10	13	0.6417
Median (pg/ml)	1.492	1.452	
Range (pg/ml)	1.359– 1.589	1.382– 1.632	
Culture supernatants	Unstimulated	+ CMV antigens	P value
n =	8	8	0.0610
Median (pg/ml)	1.459	1.552	
Range (pg/ml)	1.373– 1.569	1.432– 1.686	

Table 6.3. BAFF antigen secretion in the serum of healthy CMV- and CMV+ subjects and in culture supernatants with and without CMV antigen stimulation. No statistically significant differences were seen between the groups.

6.42. BAFF and BAFF-R Gene Expression

Expression of BAFF and BAFF-R proteins detected by flow cytometry on B cells were confirmed at the message level with qPCR experiments; B cells isolated from whole PBMCs from CMV+ subjects cultured with and without CMV antigen for 7 days were utilised for RNA extraction and thereafter cDNA synthesis. The experiment involved 5 samples from stimulated and non-stimulated B cells that were used in the qPCR reaction and the results were calculated according to Δct equation (the tested cycles were subtracted from the house keeping genes and the yielded Δct were calculated by $(2^{\Delta\text{ct}})$. The final value was then calculated as a percentage of fold increase in stimulated and non-stimulated cells. When a two tail Wilcoxon matched-pairs signed rank test was applied, a statistically significant difference was found ($P=0.0313$; Fig. 6.41). Similarly, the synthesized cDNA extracted from isolated B cells following culture with and without CMV antigens was utilised in qPCR reaction to confirm the relative gene expression of BAFF-R. The results from the reaction were calculated in the same formula above ($2^{\Delta\text{ct}}$), percentages of the fold increase were estimated and the values were compared with a one tail Wilcoxon matched-pairs signed rank test and there was statistically significant difference $P<0.05$ ($P= 0.0313$; Fig. 6.41).

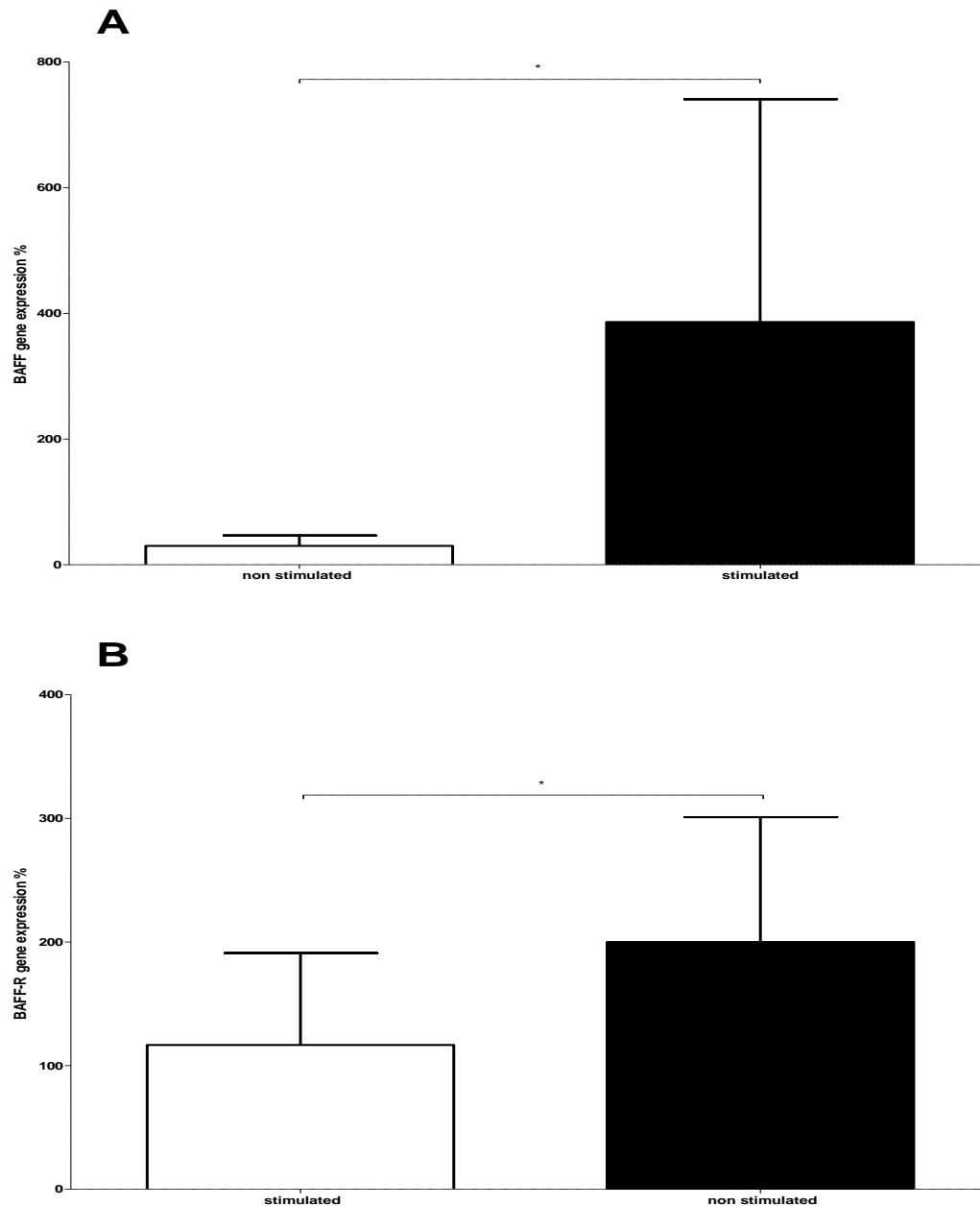


Fig. 6.41. Gene expression of BAFF (A) and BAFF-R (B) in B cells isolated from whole PBMCs from CMV+ subjects cultured with or without CMV antigen for a week, $n = 5$ for A and B, $*P < 0.05$.

6.43. Cytokines gene expression

In parallel, changes in B cell expression of other immune related genes in response to stimulation with CMV antigens was investigated; a panel of markers and cytokine

genes were used. The relative gene expression of these biomarkers in healthy subjects were evaluated with qPCR in B cells isolated from whole PBMCs cultured with or without CMV antigens for a week to test whether CMV antigens can upregulate or inhibit the production of these biomarkers during CMV infection which could play role in pathogenies or evading immune system. RNA was extracted from stimulated and non-stimulated B lymphocytes and cDNA was synthesized and later involved in qPCR reaction. Various qPCR probes were used to study the relative gene expression of TGF- β 1, IFN- γ , TNF- α , IL-6 and IL-8, details were explained in Chapter 2. PBMCs extracted from healthy subjects were cultured with and without CMV antigens for a week and RNA was separated from isolated B lymphocytes and converted to cDNA. The cDNA was used in qPCR reactions to determine the levels of TGF- β 1, IFN- γ , TNF- α , IL-6 and IL-8 gene expression; the results were calculated by subtracting the values of each cycle from housekeeping genes (Δ ct), this value then calculated via (2^{Δ ct) equation to produce the fold change compared to housekeeping genes. The fold changes between the stimulated and non-stimulated cells were then converted to percentages and the difference between the two groups was tested with a two tails Wilcoxon matched-pairs signed rank test and statistically significant differences was noted for TGF- β 1, INF- γ and TNF- α ($P < 0.01$ and $P < 0.05$; Table 6.4; Fig. 6.42).

Cytokine		Non-stimulated	CMV stimulated	P value
TGF-β1	n = 7	237.68	391.81	0.0156
INF-γ	n = 10	2.72	50.57	0.0020
TNF-α	n = 7	118.35	288.39	0.0156
IL-6	n = 5	44.55	249.97	0.0625
IL-8	n = 5	62.85	131.74	0.0625

Table 6.4. The relative gene expression of some cytokines in B cells isolated from PBMCs cultured with and without CMV antigens for a week. Significant differences between stimulated and non-stimulated B cells are shown in bold.

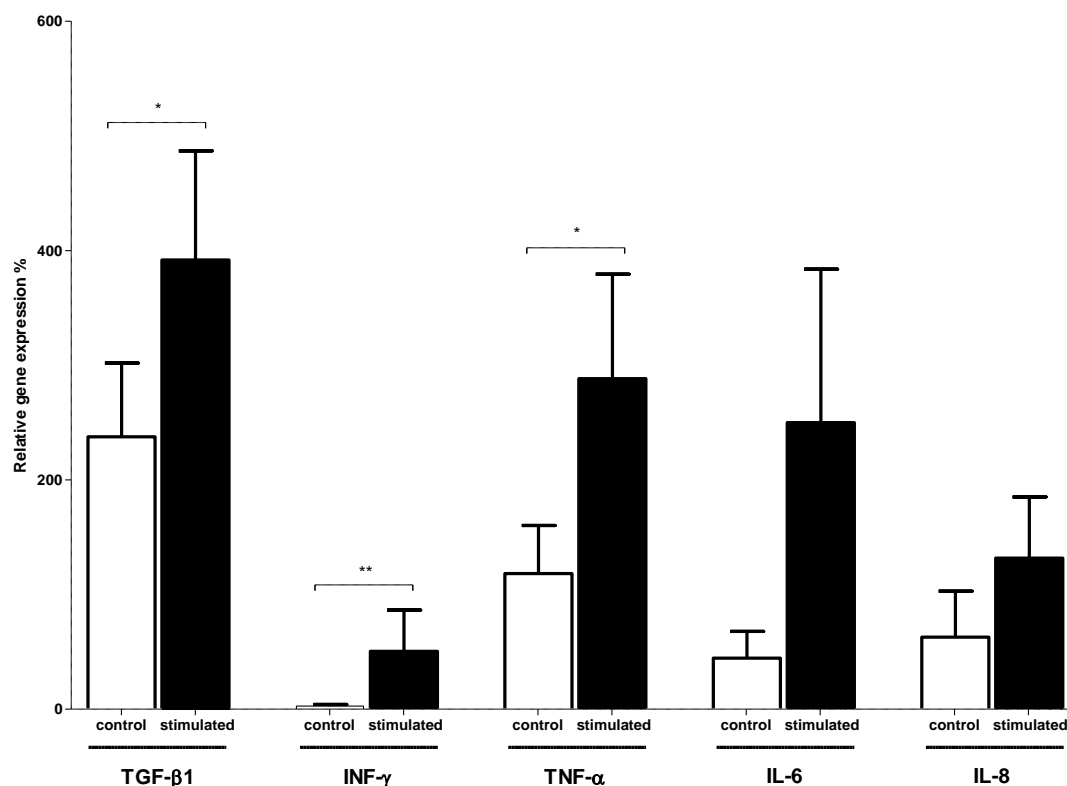


Fig. 6.42. The relative gene expression of some cytokines in B cells isolated after culture with or without CMV antigens for seven days. Empty column (non-stimulated), filled column (stimulated). Results are shown as mean \pm SEM, TGF- β 1 (n = 7), IFN- γ (n = 10), TNF- α (n = 7), IL-6 (n = 5), IL-8 (n = 5). ** P<0.01, *P<0.05.

6.44. Summary and discussion of CMV effects on BAFF, BAFF-R protein and cytokines gene expression

In net shell, BAFF protein expression by all PBMCs was not significantly upregulated still the CMV stimulated cells have shown higher proportions than non-stimulated cells. Unlike the HLA-G, the expression of HLA-G on BAFF+ PBMCs was significantly higher in all CMV induced cells than in control. In the same way, BAFF-R was found to be significantly downregulated on CD19+ lymphocytes and significantly increased in CD56+ cells. While, proportion of HLA-G+BAFF-R+ cells were significantly elevated after CMV induction, Real time PCR data have shown significant augment in BAFF and BAFF-R gene expression in B cells isolated from whole PBMCs culture treated with CMV antigens. On the other hand, Real time PCR for gene expression of

some cytokines (TGF- β , TNF- α and INF- γ) have demonstrated significant elevation after CMV treatment in B cells isolation from whole PBMCs culture Table 6.5.

Figure	Molecule	Cell population	CMV
6.34	HLA-G+BAFF+	CD3+CD4+	Not applicable
6.34	HLA-G+BAFF+	CD3+CD8+	Not applicable
6.34	HLA-G+BAFF+	CD19+	Not applicable
6.34	HLA-G+BAFF+	CD14+HLA-DR+	Not applicable
6.34	HLA-G+BAFF+	CD3+CD56+	Not applicable
6.34	HLA-G+BAFF+	CD56+	Not applicable
6.37	BAFF-R	CD19+	Not applicable
6.37	BAFF-R	CD56+	Not applicable
6.40	HLA-G+BAFF-R	CD3+CD4+	Not applicable
6.40	HLA-G+BAFF-R	CD3+CD8+	Not applicable
6.40	HLA-G+BAFF-R	CD19+	Not applicable
6.40	HLA-G+BAFF-R	CD14+HLA-DR+	Not applicable
6.40	HLA-G+BAFF-R	CD3+CD56+	Not applicable
6.40	HLA-G+BAFF-R	CD56+	Not applicable
6.41A	BAFF	B cells	Not applicable
6.41B	BAFF-R	B cells	Not applicable
6.42	TGF- β	B cells	Not applicable
6.42	TNF- α	B cells	Not applicable
6.42	INF- γ	B cells	Not applicable

Table 6.5. Summary of significant differences in proportions of BAFF, BAFF-R protein and BAFF, BAFF-R and cytokines gene expression following induction with CMV antigens.

The data presented by the current study have shown that BAFF antigen was slightly elevated in response to CMV stimulation following incubation, however, no significant increment was obtained compared to control cells. This could illustrate the effects of CMV in suppressing the expression of this protein on various cell populations in order to induce a tolerogenic microenvironment that may help in the process of eluding the effector immune cells. Albeit, the expression of BAFF and sBAFF have been to be dramatically increase in CMV positive renal transplant patients, suggesting a positive association of CMV and BAFF protein (Haiyan Xu et al., 2014). The upregulation of BAFF in such cases could induce the production of cytokines that prevent the activation of immune cells against the virus or a BAFF mediated tolerance mechanism that could spare viral recognition by immune cells may be achieved.

On the other hand, the BAFF-R has shown to be significantly down regulated of on CD19+ cells and significantly upregulation on on CD56+ lymphocytes. The receptor has also been reported to be found on T lymphocytes including T reg in addition to its abundant expression on B cells (Ye et al., 2004). A probable explanation for this receptor upregulation on NK cells could involve the induction of the NF- κ B pathway under the influence of CMV infection which could yield the receptor to be display on such cells (DeMeritt, Milford, & Yurochko, 2004), this could potentially promote the expression of BAFF-R on PBMCs including B cells. However, BAFF-R expression on B lymphocytes could have negative effects on CMV and activation of this receptor could involve BAFF expression thus improving B cell activity and defensive properties (Thibault-Espitia, Foucher, Danger, Migone, Pallier, Castagnet, G.-Gueguen, et al., 2012). This may play role in viral suppression via an antibody mediated response which ultimately lead to viral clearance. Yet, BAFF-R expression was inhibited on B cells as indicated earlier suggests a viral role in suppressing this molecule to avoid B cell mediated activation and Abs production.

On the other hand, the relative gene expression of Gene expression experiments presented in this study demonstrated a significant increment of gene expression of some cytokines including TGF- β , INF- γ , and TNF- α in response to CMV stimulation in B cells isolated from whole PBMCs culture. Such finding was also confirmed by other group who reported the that some cytokines could be potentially increase during CMV infection (Masako Shimamura et al., 2010). In the case of TGF- β gene expression which was drastically increased could have a prominent effect on inhibiting cellular

proliferation of the effector immune cells as well as on the effectiveness of the immune cells (Michelson et al., 1994a). While, increment level of INF- γ gene expression which was also significantly found to increase during viral protein induction, could be attributed to the possibility of stimulated T cells when engaging with viral antigens are promote to secrete INF- γ as part of an antiviral strategy (van den Pol et al., 2007). However, from viral point of view, INF- γ can inhibit the classical MHC I expression of on the cell surface thus hindering the ability to present viral protein for CTL (Halenius et al., 2011). This may allow viral induced HLA-G to be expressed proportionately higher, thus creating a tolerogenic microenvironment favouring surveillance escape. Likewise, TNF- α gene expression was found to be substantially augmented in CMV treated B cells, which could be as part of the role played immediate early genes of CMV that result in increased mRNA promoter activity causing increment in gene expression and thus more inhibitory cytokine translation and production (Geist et al., 1994). Such actions could be implicate in maintenance of viral activity and propagation of new virions, because it has been reported that TNF- α is vital for activating immediate early genes of CMV which can then be achieve through NF- κ B signalling pathway (Simon, Seckert, Dreis, Reddehase, & Grzimek, 2005).

CHAPTER SEVEN RESULTS

**HLA-G and KIR2DL4 expression in renal
transplant patients**

Following HLA-G and KIR2DL4 experiments in healthy subjects, these proteins were further investigated in renal transplant patients. As high HLA-G was reported to associate with less rejection incidence (Lu et al., 2011) and because KIR2DL4 possesses both inhibitory and activation functions (Vilches & Parham, 2002), it was interesting to examine expression of the two proteins in renal transplant patients. This chapter will describe the expression of HLA-G and KIR2DL4 in various PBMC populations obtained from 23 renal transplant patients before transplant and after transplantation in a consecutive manner. Also, the levels of sHLA-G will be assessed in serum samples separated from those patients pre transplant and after transplantation to study the association of this marker with the transplant process. In addition, the occurrence of the 14bp insertion/deletion dimorphism and SNPs in exon 8 of HLA-G will be evaluated to establish any correlation with transplant outcome. Also, KIR2DL4 genotyping will be assessed in relation to KIR2DL4 expression and to see whether the KIR2DL4 genotype correlated with high levels of the protein expression.

7.1. Renal transplant patients

The purpose of this study was to assess the correlation of HLA-G expression and its ligand, KIR2DL4, in patients eligible for renal transplantation. The study was designed to obtain sequential blood samples, pre-transplant, 2 months, 4 months and 6 months post transplantation. A cohort of 39 renal transplant patients were recruited in Royal Liverpool University Hospital during 2015 and 2016 after obtaining ethical approval. Blood samples (7-10ml) were taken from each anonymised participant after obtaining written consent. The blood was processed as soon as samples were received and mononuclear cells were isolated via gradient centrifugation with Ficoll solution, at the same time, plasma was separated and frozen at -20 °C to be further utilised for sHLA-G assessment. PBMCs extracted from all patients were labelled with a panel of mAbs specific for HLA-G and KIR2DL4 as well as with basic PBMC subpopulation markers. Data were acquired with Accuri C6 flow cytometry and analysed with the software provided. The same pattern of antibody labelling was performed for each patient pre-transplant and then after renal transplantation whenever a sample was obtained.

7.2. HLA-G expression by CD3+CD4+CD45RA+ cells pre and post renal transplantation

HLA-G expression was assessed on CD3+CD4+CD45RA+ cells before and after transplantation at several time points. There was a moderate increase in the proportion of HLA-G+ cells especially at 2 months post transplantation (6.09%) then there was slight drop at 4 months (3.81%) and slight increment at 6 months (4.97%) compared to the pre-transplant percentage 0.13%. After applying repeated Measures ANOVA, Dunnett's Multiple Comparison Test, the overall $P=0.2478$ (Fig. 7.1a). Some flow cytometry plots for HLA-G expression is revealed in (Fig. 7.1b)

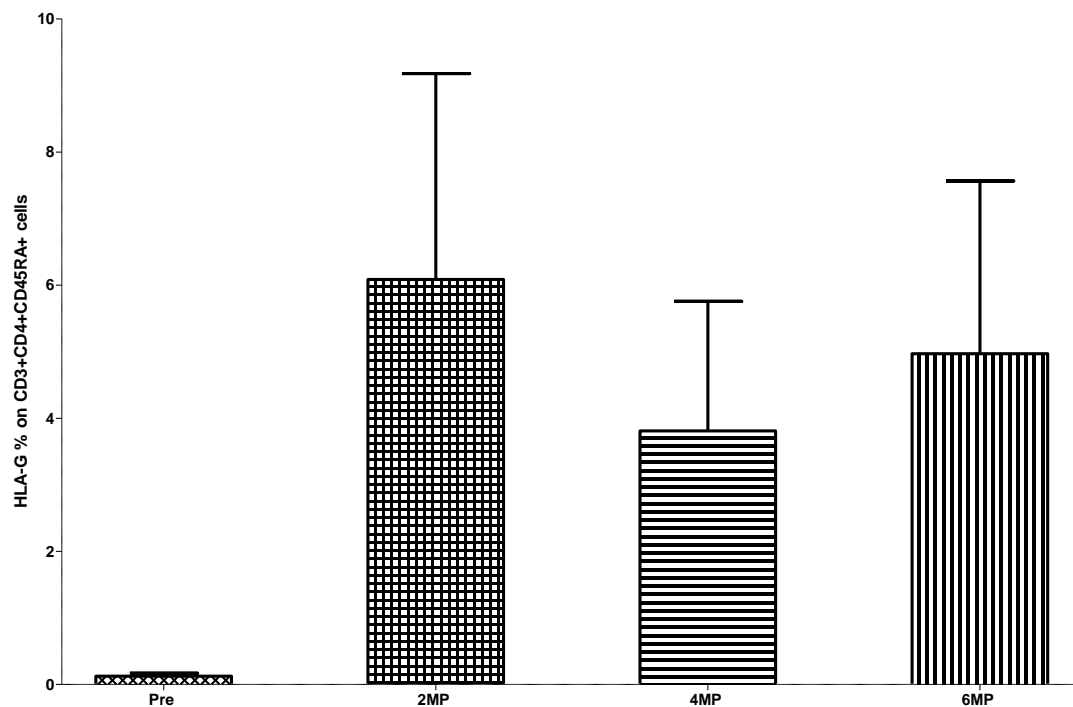


Fig. 7.1a. The expression of HLA-G on CD3+CD4+CD45RA+ lymphocytes before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $P > 0.05$.

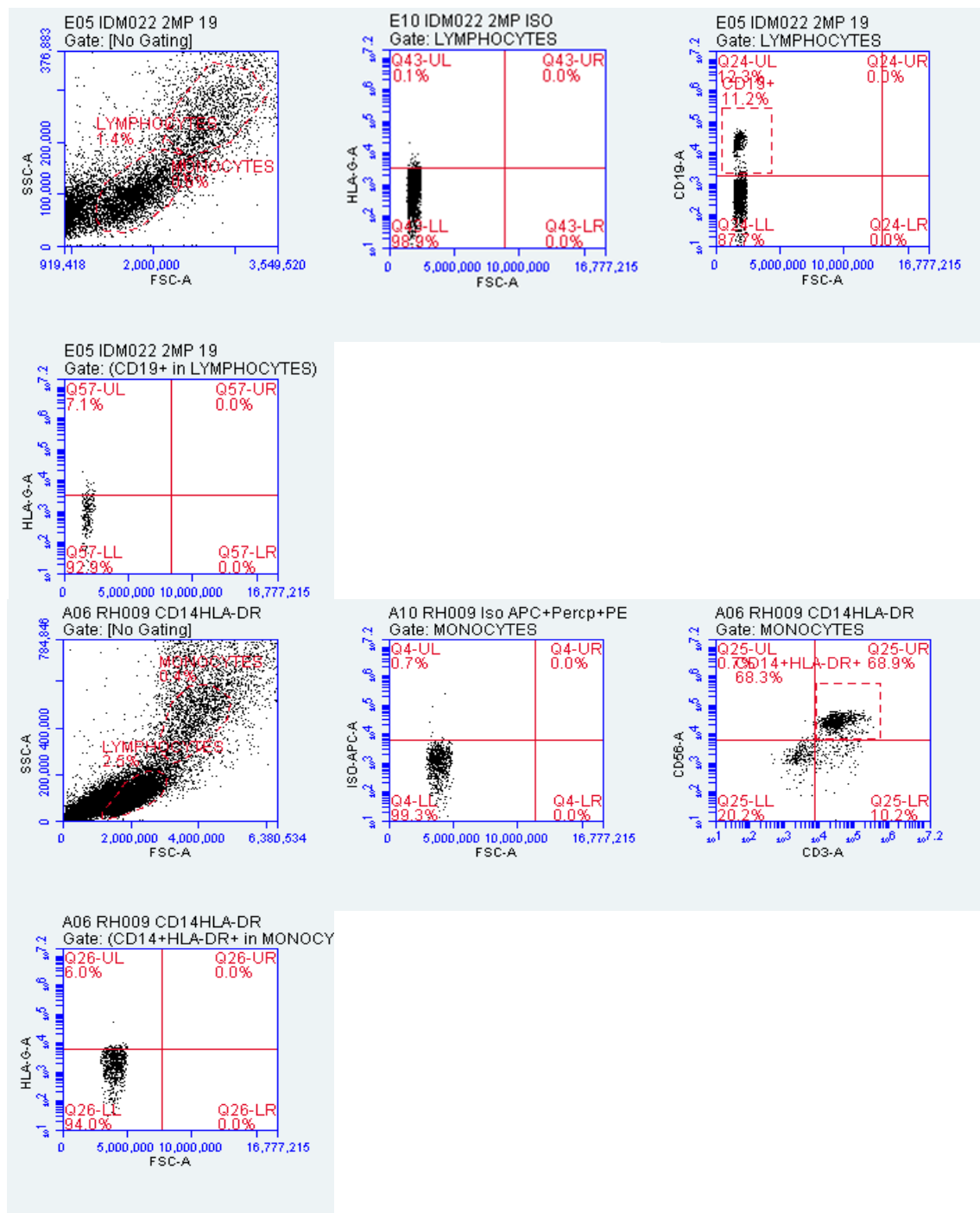


Fig. 7.1b. Flow cytometry acquisition plots displaying lymphocytes (top panel) and monocytes (lower panel) gating in renal transplant patients (two months post transplantation) and further co-labelling of CD19 and HLA-G for lymphocytes and CD14 as well as HLA-DR for monocytes in addition to isotype labelling in both panels.

7.3. HLA-G expression by CD3+CD4+CD45RO+ cells pre and post renal transplantation

The expression of HLA-G on PBMCs labelled with CD3+CD4+CD45RO+ was evaluated at sequential time points before and after renal transplantation (Fig. 7.2) and the proportion of HLA-G was highest after 2 months of transplantation (4.81%) compared to 0.43% before transplantation. The HLA-G proportion was slightly lowered at 4 months (2.98%) and then increased at 6 months 3.59%. No statistically significant difference was recorded with overall P value =0.0638 after a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test was performed, but there was a significant difference between pre and 2 months post transplantation (*P<0.05).

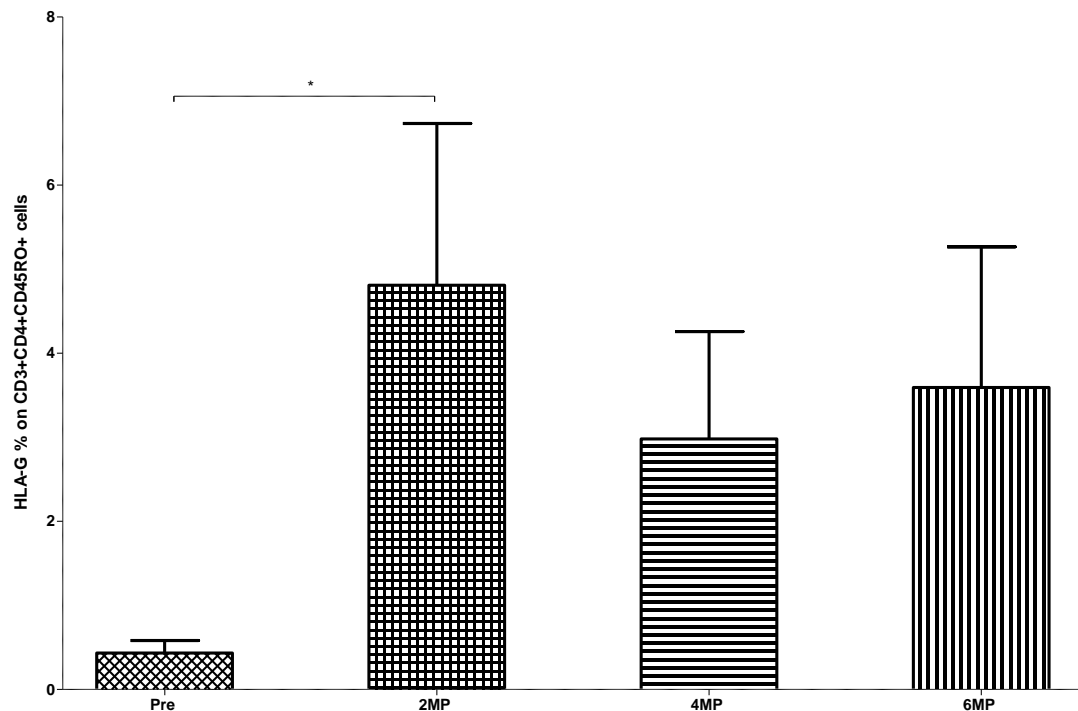


Fig. 7.2. The expression of HLA-G on CD3+CD4+CD45RO+ lymphocytes before and after renal transplantation. Results are expressed as mean \pm SEM, n = 13, P>0.05.

7.4. HLA-G expression by CD3+CD8+CD45RA+ cells pre and post renal transplantation

The expression of HLA-G was assessed on CD3+CD8+CD45RA+ on a sequential basis (Fig. 7.3) and as illustrated there was gradual increase in the proportion of HLA-G expression post transplantation (pre =0.81%, 2 months =1.62%, 4 months = 2.208% and 6 months = 2.44%). Upon performing a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test, the overall P= 0.5223 and no statistically significant difference was recorded.

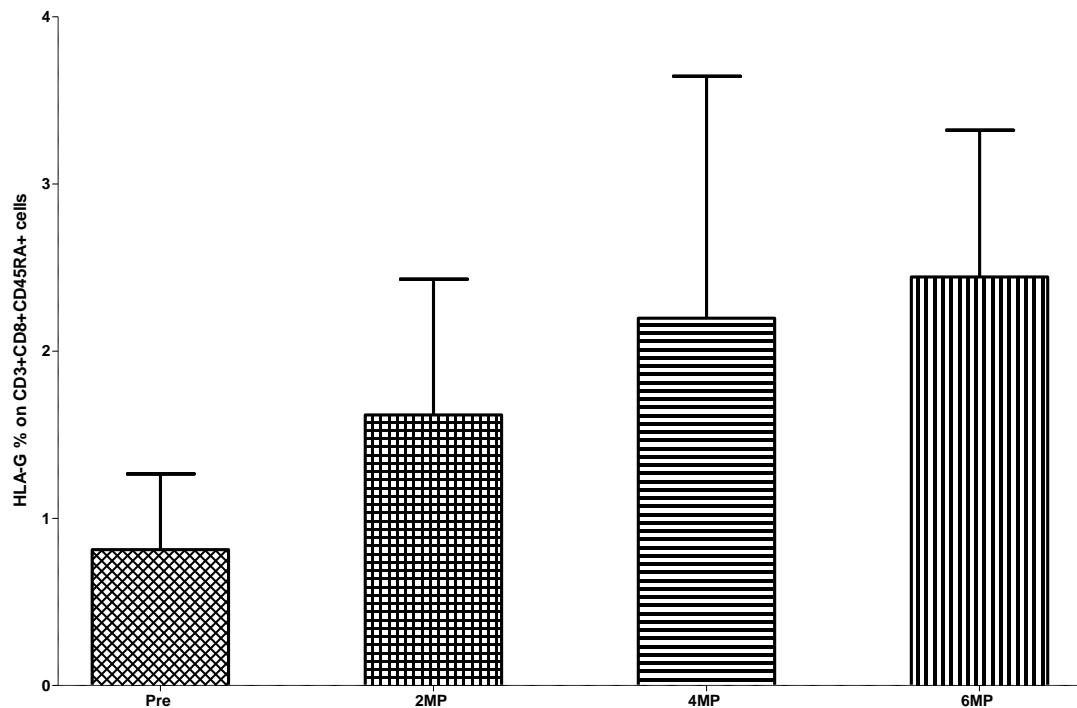


Fig. 7.3. The expression of HLA-G on CD3+CD8+CD45RA+ lymphocytes before and after renal transplantation. Results are expressed as mean \pm SEM, n = 13, P>0.05.

7.5. Expression of HLA-G by CD3+CD8+CD45RO+ cells pre and post renal transplantation

In the same way, HLA-G expressed by CD3+CD8+CD45RO+ cells was measured before and after transplantation (Fig. 7.4) and the proportion was highest at 2 months following transplantation (7.01%) compared to the pre-transplant proportion (2.38%). Then after 2 months the percentage of HLA-G was gradually dropping at 4 months (5.19%) and 4.20% at 6 months. When a Repeated Measures

ANOVA, Dunnett's Multiple Comparison Test, was applied there was no statistically significant difference recorded with overall $P = 0.5082$.

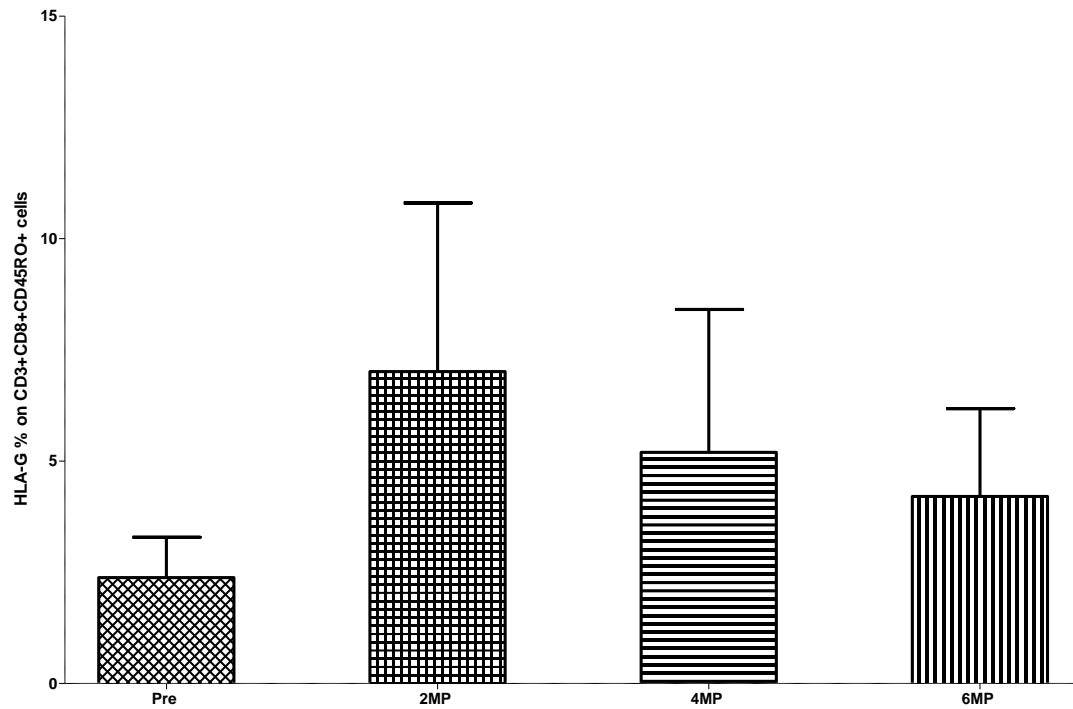


Fig. 7.4. The expression of HLA-G on CD3+CD8+CD45RO+ lymphocytes before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $P > 0.05$.

7.6. HLA-G expression by CD3+CD4+CD69+ cells pre and post renal transplantation

Likewise, the HLA-G was assessed on CD3+CD4+CD69+ cells at consecutive points before and after transplantation (Fig. 7.5) and it was shown that the proportion of HLA-G+ cells was increased at 2 months (12.51%) compared to 3.36% before transplantation. Then after, at 4 months it was 7.38% and the proportion increased to 10.59% after 6 months. No statistically significant difference was obtained when a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test, was applied with overall ($P = 0.0706$). However, there was significant difference between pre and 2 months post transplant ($* P < 0.05$).

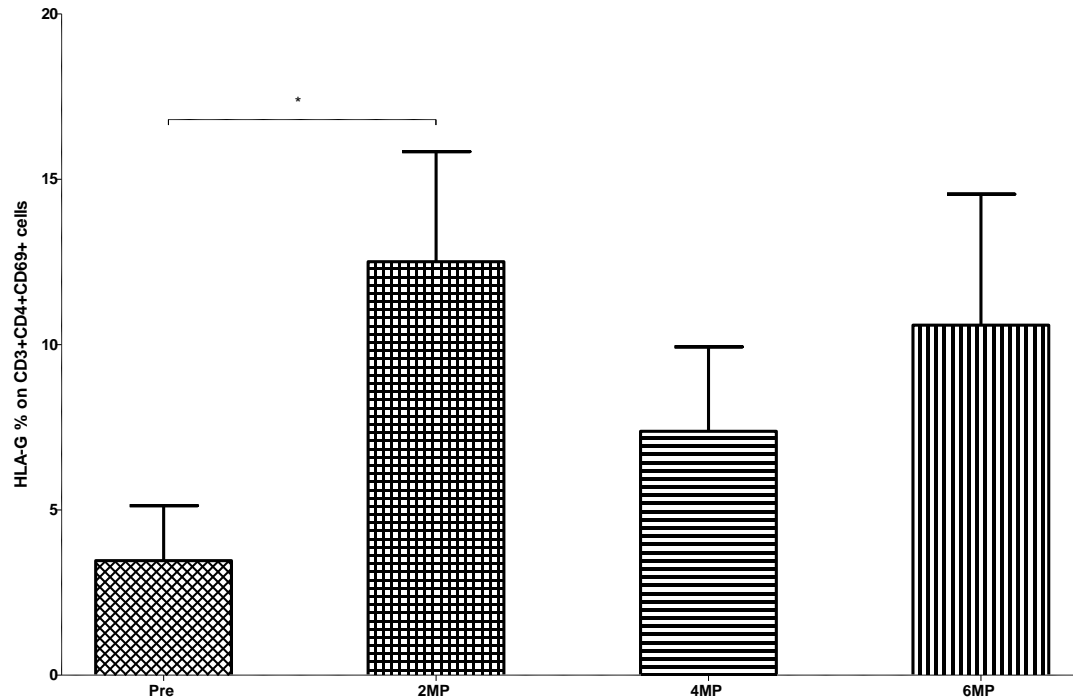


Fig. 7.5. The expression of HLA-G on CD3+CD4+CD69+ lymphocytes before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $*P < 0.05$.

7.7. Expression of HLA-G by CD3+CD4+ cells pre and post renal transplantation

Similar to the above approach, the HLA-G was measured on CD3+CD4+ lymphocytes at pre-and post-transplantation (Fig. 7.6) and the proportion was highest at 2 months post 5.71% compared to 0.56% pre-transplant. At 4 months post, there was a slight decrease in the HLA-G expression to 2.62% and the proportion was slightly increased at 6 months post (4.98%). Upon performing a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test, there was no statistically significant difference noted with overall $P = 0.0932$.

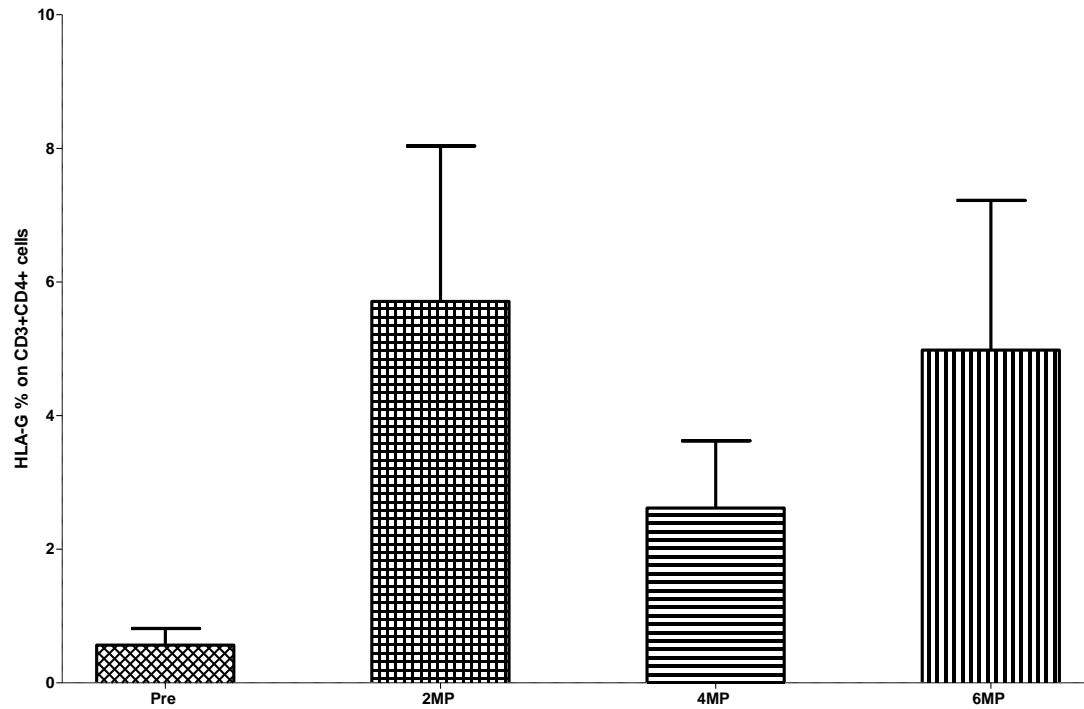


Fig. 7.6. The expression of HLA-G on CD3+CD4+ lymphocytes before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $P > 0.05$.

7.8. HLA-G expression in CD3+CD8+CD69+ lymphocytes pre and post renal transplantation

The expression of HLA-G on CD3+CD8+CD69+ cells were recorded before and after transplantation as shown in (Fig. 7.7) and the base line of HLA-G pre-transplantation was 10.72%, then there was slight increase at 2 months post 12.57% after that the proportion declined at 4 months and 6 months (9.83% and 8.57%) respectively. No statistically significant difference was observed after applying a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test, with overall $P = 0.9417$.

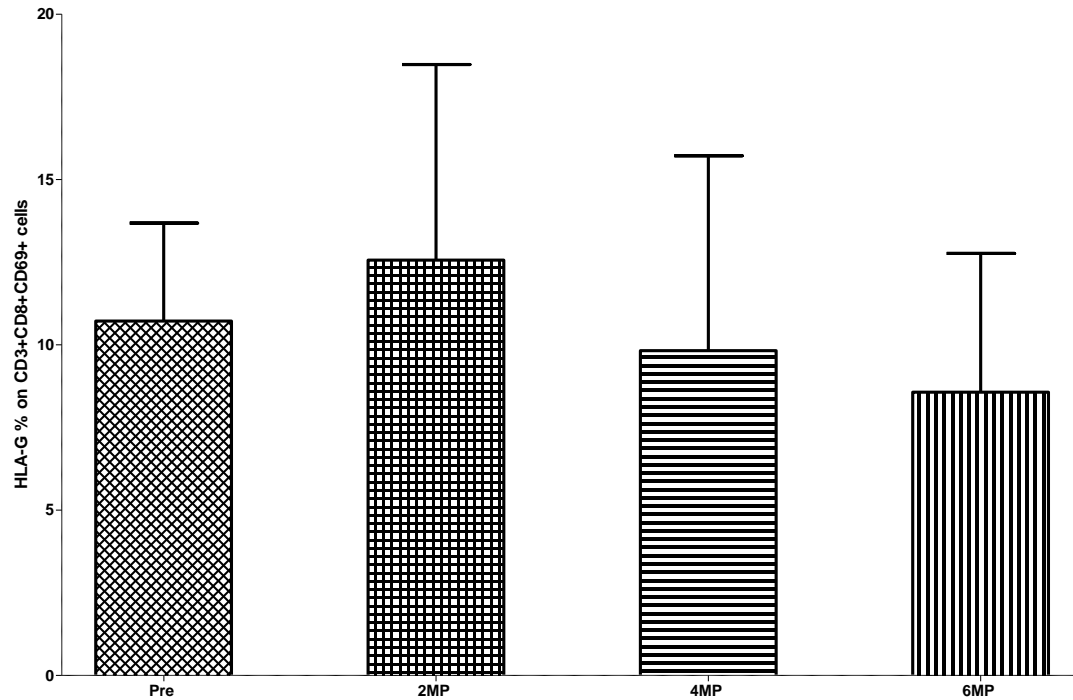


Fig. 7.7. The expression of HLA-G on CD3+CD8+CD69+ lymphocytes before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $P > 0.05$.

7.9. Expression of HLA-G by CD3+CD8+ lymphocytes pre and post renal transplantation

A similar pattern was followed with CD3+CD8+ cells; HLA-G was assessed before and after transplantation (Fig. 7.8) and at 2 months post the proportion of HLA-G was highest (4.39%) compared to pre-transplant (1.06%) while at 4 and 6 months post there were drops in the HLA-G proportion (2.757% and 2.871%) respectively. No statistically significant difference was obtained when a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test was applied with overall $P = 0.5744$.

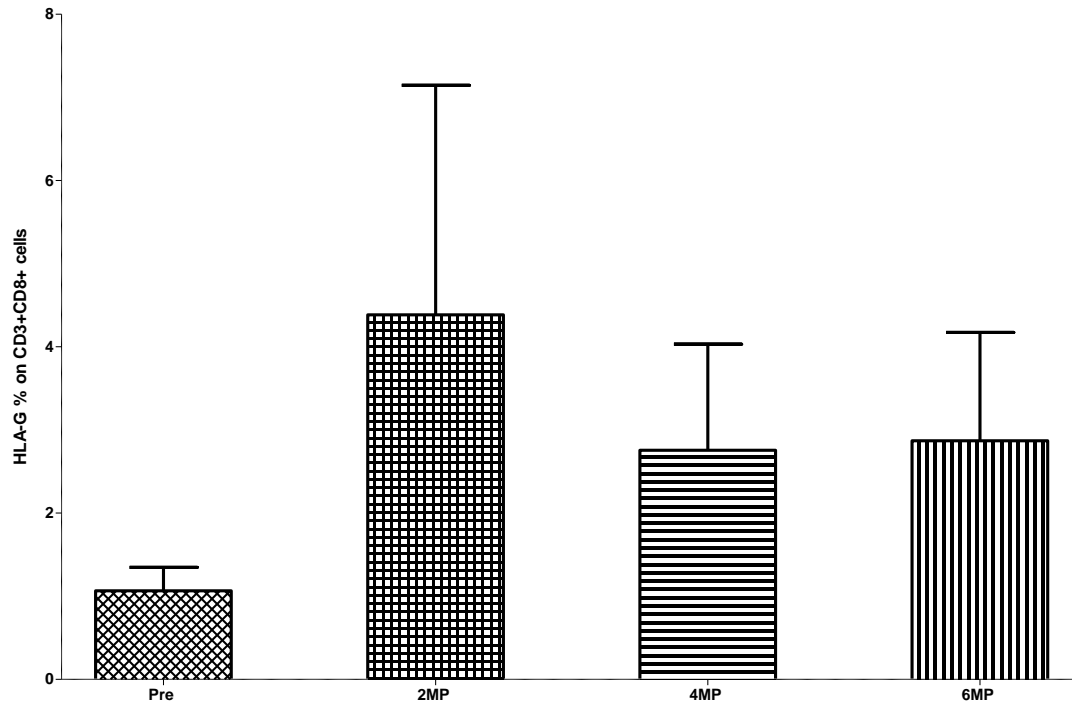


Fig. 7.8. The expression of HLA-G on CD3+CD8+ lymphocytes before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $P > 0.05$.

7.10. Expression of HLA-G by CD3+CD56+ lymphocytes pre and post renal transplantation

Unlike other PBMC populations, CD3+CD56+ cells showed the highest HLA-G proportion at pre-and 4 months following transplantation (17.44% and 17.01%) respectively, while, there was a slight drop in the HLA-G proportion at 2 and 6 months (14.83% and 15.83%) respectively (Fig. 7.9). No statistically significant difference was obtained after a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test was performed, overall $P = 0.9818$.

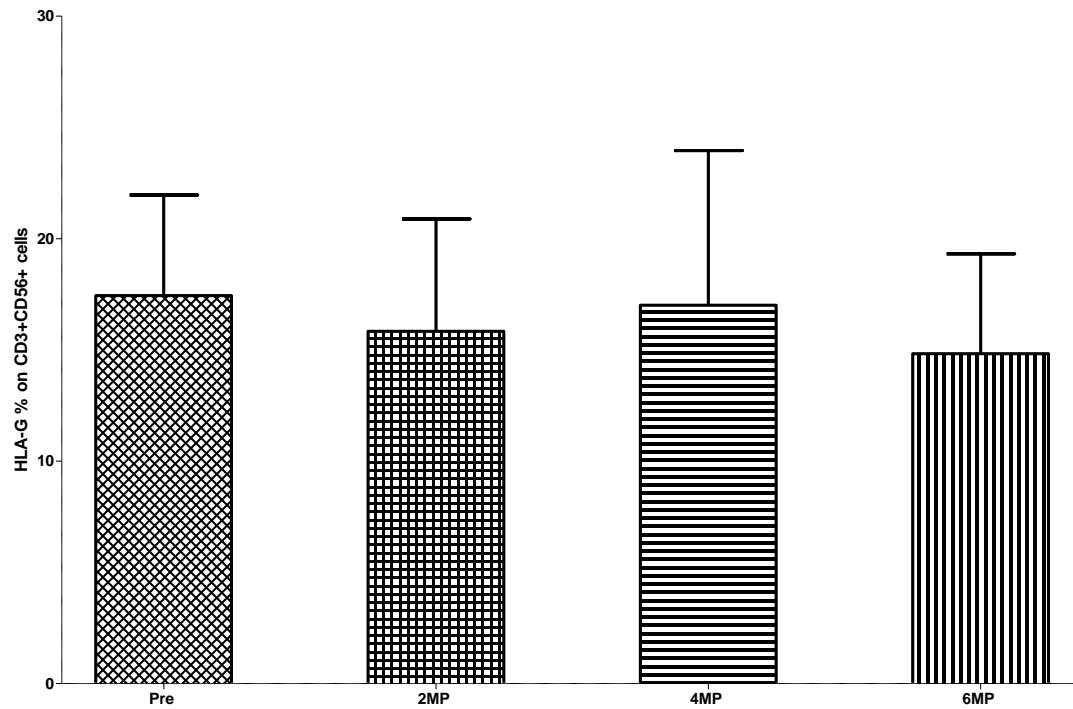


Fig. 7.9. The expression of HLA-G on CD3+CD56+ lymphocytes before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $P > 0.05$.

7.11. HLA-G expression by CD56+ lymphocytes pre and post renal transplantation

In CD56+ cells the expression of HLA-G showed elevated proportions after 6 months of transplantation (3.68%) compared to the pre-transplant proportion, 0.92% (Fig. 7.10), while at 2 months the proportion was 0.82% and slightly increased at 4 months post transplantation to 1.25%. When a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test was applied, no statistically significant difference was obtained with overall $P = 0.1109$.

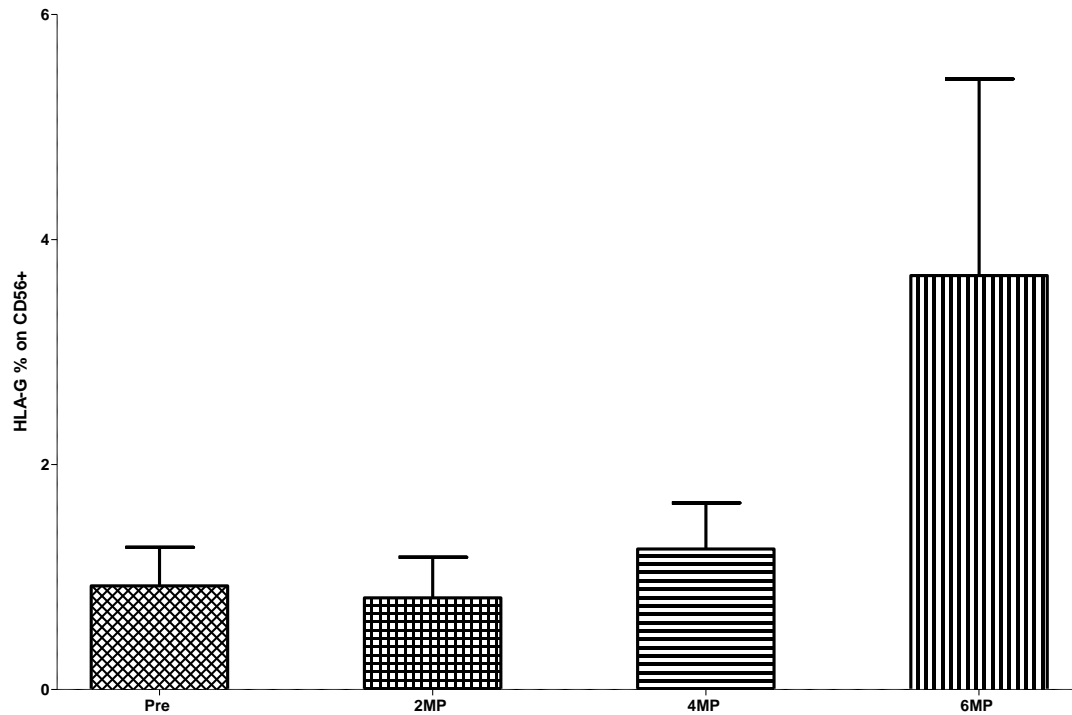


Fig. 7.10. The expression of HLA-G on CD56+ cells before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $P > 0.05$.

7.12. Expression HLA-G by CD19+ lymphocytes pre and post renal transplantation

In CD19+ cells the HLA-G expression before transplantation was recorded at 1.57% and the proportion declined at 2 and 4 months respectively (0.84% and 0.80%) then at 6 months post transplantation there was slight increase with an HLA-G+ proportion of 2.21%. No statistically significant difference was obtained after a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test was performed with overall $P = 0.2080$ (Fig. 7.11).

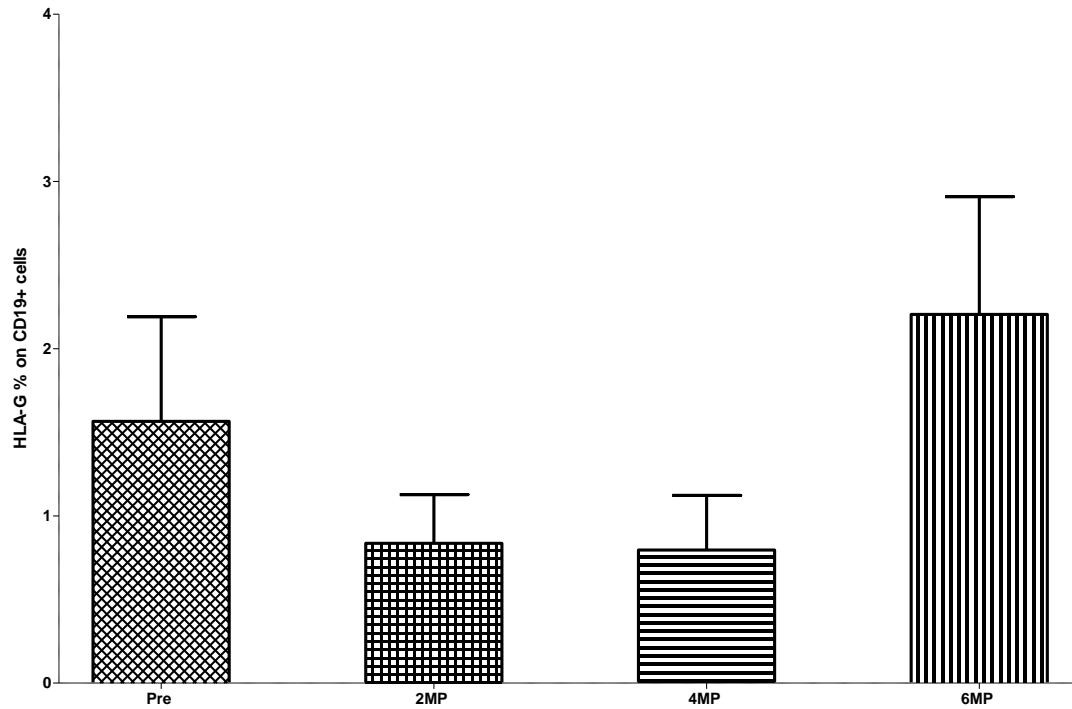


Fig. 7.11. The expression of HLA-G on CD19+ lymphocytes before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $P > 0.05$.

7.13. Expression of HLA-G by CD14+HLA-DR+ cells pre and post renal transplantation

Finally, the expression of HLA-G on CD14+HLA-DR+ was tested in the same patients before and after transplantation (Fig. 7.12) and the pre-transplant proportion of 5.54% was higher than at 2 and 4 months post transplantation, 3.78% and 3.02% respectively but at 6 months post the proportion of HLA-G+ cells was doubled to 12.04%. There a was statistically significant difference obtained after applying a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test, with overall $P = 0.0063$. In particular, a significant difference was found between pre and 6 months post transplantation ($P < 0.05$).

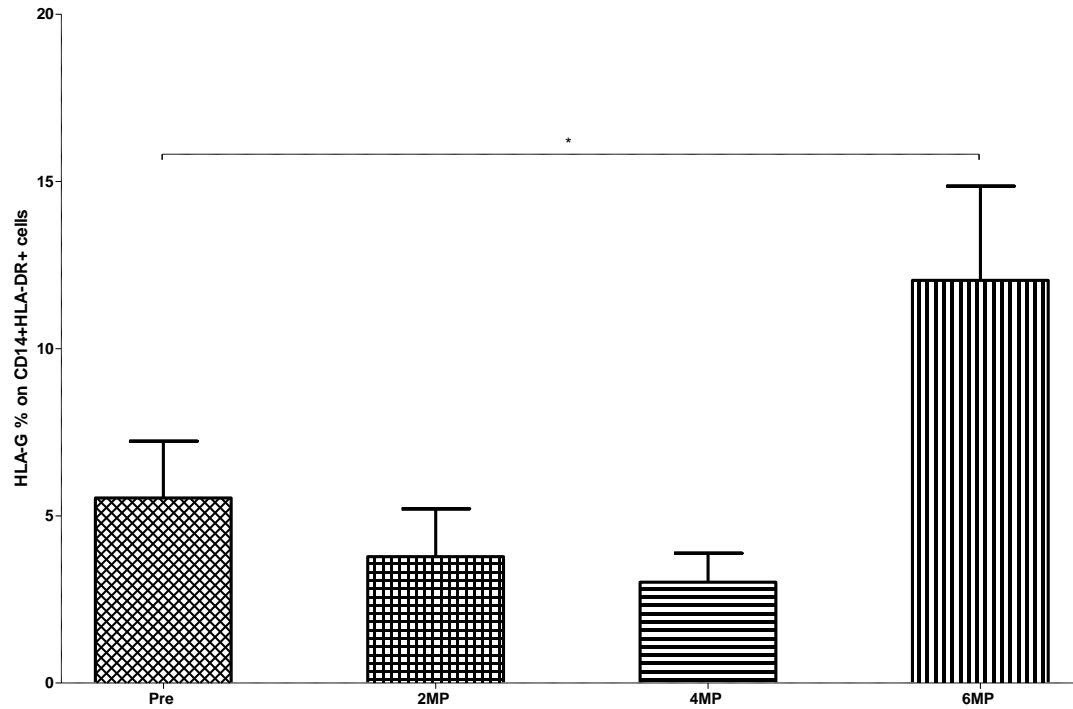


Fig. 7.12. The expression of HLA-G on CD14+HLA-DR+ cells before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $*P < 0.05$.

7.14. Expression of HLA-G by various PBMC populations pre and 2 months post renal transplantation

A group of 10 renal transplant patients who were involved in the current study were only able to provide 2 blood samples (before and 2 months after transplantation). The PBMCs were extracted and labelled with monoclonal antibodies specific for HLA-G and KIR2DL4 as well as group specific antibodies. Data obtained from Accuri C6 Flow cytometry were analysed and data were plotted in Fig. 7.13. In general, there was marked elevation in the proportion of HLA-G expressing cells in all the tested cell populations after 2 months of transplantation (Table 7.1). When the groups were compared with a Paired t test (two tailed) there was no statistically significant difference ($P > 0.05$) among almost all the tested groups apart from CD19+ lymphocytes which showed statistically significant difference 2 months after transplantation $P = 0.0496$.

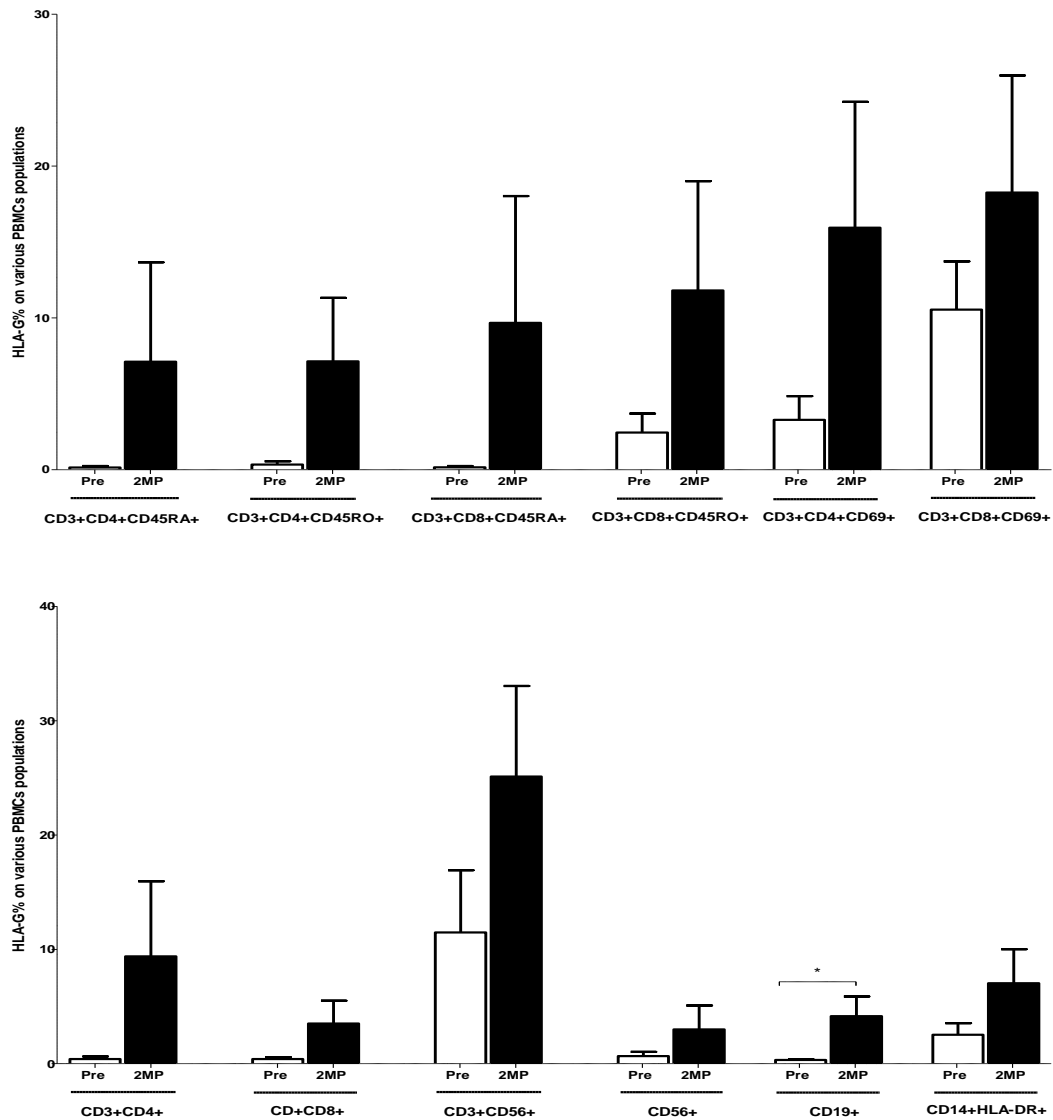


Fig. 7.13. The expression of HLA-G on PBMC populations pre and 2 months after renal transplantation. Results are expressed as mean \pm SEM, n = 10, *P<0.05.

Mean HLA-G% on various PBMC populations			
PBMCs	Pre-transplant	2 months post	P value
CD3+CD4+CD45RA+	0.1480	7.123	0.3152
CD3+CD4+CD45RO+	0.3440	7.142	0.1404
CD3+CD8+CD45RA+	0.1640	9.676	0.2830

CD3+CD8+CD45RO+	2.448	11.81	0.2381
CD3+CD4+CD69+	3.285	15.95	0.1463
CD3+CD4+	0.4090	9.388	0.1978
CD3+CD8+CD69+	10.55	18.26	0.2821
CD3+CD8+	0.4180	3.512	0.1419
CD3+CD56+	11.48	25.11	0.1294
CD56+	0.6780	2.995	0.3262
CD19+	0.3280	4.164	*0.0489
CD14+HLA-DR+	2.529	7.038	0.2231

Table 7.1. Paired t test comparing HLA-G expression on various PBMCs before and 2 months after transplantation, n=7, *P<0.05

7.15. Quantitation of sHLA-G in renal transplant patients

Plasma samples extracted from whole blood of renal transplant patients were assessed with a sHLA-G ELISA (Table 7.2). The samples from pre-transplant as well as 2 months and 6 months post transplantation were included in the run. The mean concentrations for sHLA-G were compared with a repeated measures ANOVA (Tukey's Multiple Comparison Test) but no statistical difference was obtained (P=0.1287). In the same way, archived sera obtained from renal transplant patients at the Royal Liverpool University Hospital were assessed with sHLA-G ELISA, three successive time points were chosen (pre, 6 months and 12 months post transplantation). Upon comparing the concentrations of sHLA-G with a repeated measures ANOVA (Tukey's Multiple Comparison Test), no statistically significant difference was noted (P=0.1089).

Serum	Pre	2 months post	6 months post	P value
n =	10	10	10	0.1287
Median (U/ml)	142.59	144.54	245.80	

Range (U/ml)	59.4-1255.17	25.39-573.92	49.21-969.0	
Mean (U/ml)	249.97	188.23	291.31	
Serum	Pre	6 months post	12 months post	P value
n =	20	20	20	0.1089
Median (U/ml)	38.68	22.77	49.43	
Range (U/ml)	14.63-933.07	7.44-131.39	13.81-381.37	
Mean (U/ml)	108.670	45.212	99.693	

Table. 7.2. Levels of soluble HLA-G (units/ml) in serum from renal transplant patients measured at three-time points (Upper Panel, pre, 2 months and 6 months post transplantation). Also, sHLA-G was tested in archived sera from renal transplant patients (Lower Panel, pre, 6 months and 12 months post transplantation) by sHLA-G ELISA. $P>0.05$.

7.16. HLA-G 14 bp insertion/deletion genotyping

Hviid et al, 2003 described the influence of a 14bp sequence in exon 8 of the HLA-G gene on HLA-G expression and genotyping of HLA-G was carried out to assess the correlation between the 14bp dimorphisms and the proportions of HLA-G expressed on various transplant patient PBMC populations in relation to CMV status. DNA was extracted from 34 renal transplant patients involved in the current study and data were obtained either from sequencing or gel electrophoresis of amplified exon 8. The frequency of (-/+) heterozygotes (41.17%) was highest among renal transplant patients and the occurrence of (-/-) homozygote ranked second (32.35%), whereas, (+/+) homozygotes were the least frequent (26.47%). The χ^2 value for heterogeneity between CMV+ and CMV- patients was not significant ($P=0.9039$; Table 7.3).

CMV status	+/-	+/+	-/-	Total
CMV +	7	5	5	17
CMV -	7	4	6	17

Table 7.3. The occurrence of the 14 bp in exon 8 of HLA-G. Insertion (+) and deletion (-) in renal transplant patients CMV+ (n = 17) and CMV- (n = 17). Differences were not statistically significant (χ^2 test; $P>0.05$).

7.17. HLA-G expression according to HLA-G Genotype

The proportion of HLA-G-expressing cells among CD3+CD56+ cells was assessed in relation to the 14bp dimorphism of the HLA-G gene. A linear regression model was applied and there was no significant correlation ($P=0.351$) between the frequency of the polymorphic patterns (ins/ins, ins/del and del/del) and proportions of HLA-G+ CD3+CD56+ cells (Table 7.4).

Linear regression HLA-G% on CD3+CD56+ with 14bp	P value
Pearson Correlation Sig. (1-tailed)	0.351

Table 7.4. Linear regression association between the proportion of HLA-G+CD3+CD56+ cells and the frequency of the 14 bp dimorphism, n = 21, $P>0.05$.

7.17.1. Prevalence of SNPs in exon 8 of the HLA-G gene and the proportion of HLA-G expressing CD3+CD56+ lymphocytes

Individual SNPs in exon 8 of the HLA-G gene have been associated with high or low expression of HLA-G protein. These SNPs were evaluated by sequencing DNA samples from renal transplant patients involved in this study. These SNPs were identified in the DNA segment when compared with a standard human DNA sequence of exon 8 of HLA-G (data available in NCBI.GOV.UK). Seven SNPs were recognised: C/T +3003, G/C +3010, C/A +3027, C/T +3035, C/G +3142, A/G +3187, C/G +3196 in exon 8 and each one will have two peaks in the sequencing traces at each site in heterozygotes. The frequencies of these SNPs were assessed in relation to HLA-G expression on CD3+CD56+ cells (Table 7.5), where linear regression revealed no significant correlation between all the SNPs and the proportions of HLA-G-expressing CD3+CD56+ cells with $P>0.05$.

Linear regression HLA-G% on CD3+CD56+ with C/T+3003	P value
Pearson Correlation Sig. (1-tailed)	0.419

Linear regression HLA-G% on CD3+CD56+ with G/C+3010	
Pearson Correlation Sig. (1-tailed)	0.340
Linear regression HLA-G% on CD3+CD56+ with C/A+3027	
Pearson Correlation Sig. (1-tailed)	0.189
Linear regression HLA-G% on CD3+CD56+ with C/T+3035	
Pearson Correlation Sig. (1-tailed)	0.189
Linear regression HLA-G% on CD3+CD56+ with C/G+3142	
Pearson Correlation Sig. (1-tailed)	0.340
Linear regression HLA-G% on CD3+CD56+ with A/G+3187	
Pearson Correlation Sig. (1-tailed)	0.122
Linear regression HLA-G% on CD3+CD56+ with C/G+3196	
Pearson Correlation Sig. (1-tailed)	0.286

Table 7.5. association between the proportion of HLA-G-expressing CD3+CD56+ cells and the frequency of SNPs in exon 8, n = 21, Linear regression, P>0.05.

7.17.2. Scoring of HLA-G expression by CD3+CD56+ cells according to the 14bp, +3142C and +3187G frequencies

Additionally, the potential influence of exon 8 SNPs on HLA-G expression was assessed via a scoring model. Alleles associated with high HLA-G expression: 14bp del, +3142C and +3187G were allocated a score of 1 while those associated with low HLA-G expression: 14bp ins, +3142G and 3187A were allocated a score of 0. Taking into consideration both copies of the HLA-G gene, this permitted each patient to be allocated a score between 0 and 6 indicative of potential for low or high expression. Expression of HLA-G on CD3+CD56+ lymphocytes in renal transplant patients was compared to these scores in Table 7.6 and Fig. 7.14. When linear regression was performed to test the association between the scores and HLA-G on CD3+CD56+ pre and 2 months post, there was no association found with P>0.05 (Table 7.7).

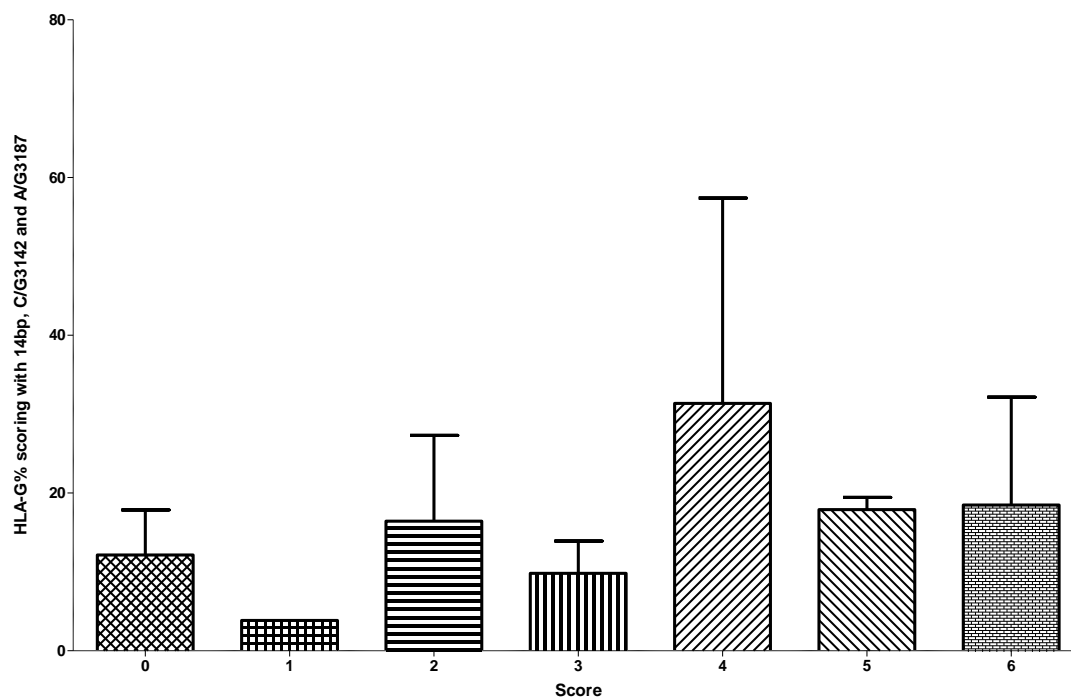


Fig. 7.14. Scoring of HLA-G alleles in relation to HLA-G expression by CD3+CD56+ cells in 23 renal transplant patients according to the 14 bp insertion/deletion, C/G +3142 and A/G +3178 dimorphisms. Results are expressed as mean \pm SEM. Columns without error bars indicate a single sample.

No.	14bp +/-	C/G +3142	A/G +3187	Score	D CMV	R CMV	Viremia	Rejection episodes	HLA-G %
1	0	0	0	0	+	+	-	-	7.18
2	0	0	0	0	-	-	-	-	0.81
3	0	0	0	0	-	-	-	-	0
4	1	1	0	2	-	+	+	-	1.93
5	0	0	0	0	-	+	-	-	3.14
6	2	1	1	4	-	-	-	-	57.4
7	1	1	0	2	-	-	-	-	6.69
8	2	1	0	3	+	+	+	-	6.05
9	1	1	1	3	+	-	-	-	20.68
10	2	1	0	3	+	+	+	-	10.95

11	0	0	0	0	+	+	-	-	43.29
12	2	1	1	4	+	+	-	-	5.32
13	0	0	0	0	+	-	-	-	1.18
14	2	2	2	6	-	-	-	-	32.16
15	0	0	0	0	+	+	-	-	30.9
16	2	2	2	6	-	-	-	-	4.81
17	1	1	0	2	+	-	-	-	48.79
18	1	1	1	3	+	+	-	-	1.6
19	2	2	1	5	+	-	-	-	19.44
20	2	2	1	5	-	-	+	-	16.36
21	1	0	0	1	-	+	-	-	3.84
22	0	0	0	0	-	+	+	+	10.66
23	1	1	0	2	-	+	-	-	8.35

Table. 7.6. Scoring the 14 bp insertion/deletion polymorphisms (0=+/, 1=+/- and 2=-/-) with C/G +3142 (0=G/G, 1=C/G and 2=C/C) and A/G +3187 (0=A/A, 1=A/G and 2=G/G) with HLA-G expression on CD3+CD56+ cells in a panel of 23 renal transplant patients. Data for CMV status of donor and recipient, the occurrence of CMV viraemia and rejection episodes are also shown.

Pearson Correlation of HLA-G% on CD3+CD56+ cells pre-transplant	P value
Sig. (1-tailed)	
SCORE	0.186
POLYMORPHISM	0.209
G/C3142	0.217
A/C3187	0.191
Pearson Correlation of HLA-G% on CD3+CD56+ cells 2 months post	P value

Sig. (1-tailed)	
SCORE	0.473
POLYMORPHISM	0.427
G/C3142	0.437
A/C3187	0.438

Table. 7.7. Association between %HLA-G+D3+CD56+ cells pre and 2 months post transplantation with scoring of the 14 bp insertion/deletion polymorphisms (0=+/, 1=+/- and 2=-/-) with C/G +3142 (0=G/G, 1=C/G and 2=C/C) and A/G +3187 (0=A/A, 1=A/G and 2=G/G) with HLA-G expression on CD3+CD56+ in a panel of 23 renal transplant patients.

7.17.3. The association of HLA-G genotyping with CMV viremia and rejection episodes

The association of the 14bp dimorphism of the HLA-G gene with CMV viremia and rejection episodes in renal transplant patients was tested through the application of logistic regression between these variables. The test revealed no significant association between the 14bp alleles and both CMV viremia and rejection incidence with $P>0.05$. Likewise, no significant association was noted between donor/recipient CMV status and both rejection episodes and CMV viremia, $P>0.05$ (Tables 7.6 and 7.8).

Logistic regression	P value
CMV episodes with 14bp polymorphism	0.892
Rejection episodes with 14bp polymorphism	0.998
D/R CMV status with CMV viremia	0.886
D/R CMV status with rejection episodes	0.504

Table 7.8. Association of 14bp dimorphism with CMV status, viraemia and rejection episodes in renal transplant patients, $n = 21$, $P>0.05$. Also, similar association was made between D/R CMV status and CMV viremia and rejection incidence, $n = 21$, $P>0.05$, logistic regression.

7.18. Expression of KIR2DL4 by CD3+CD56+ cells pre and post renal transplantation

In the patients involved in the current study, KIR2DL4 was tested before and after renal transplantation on various PBMC subpopulations using Accuri C6 Flow cytometry. The proportion of CD3+CD56+ cells expressing this protein was highest after 2 months of transplantation (16.04%) compared to (8.58%) before transplantation. The proportion then decreased gradually 4 and 6 months later (13.86% and 10.85%) respectively. When a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test was applied, no statistically significant difference was obtained ($P=0.5482$; Fig. 7.15a). Some flow cytometry plots are for lymphocytes gating and further KIR2DL4 expression are displayed in (Fig. 7.15b).

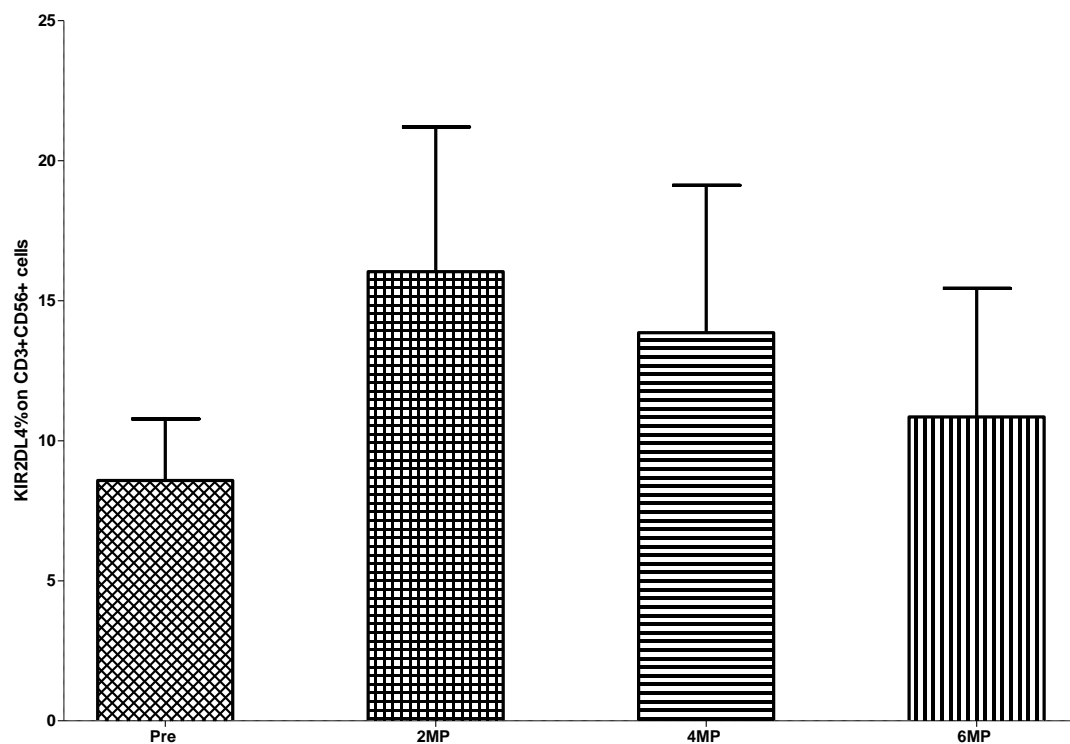


Fig. 7.15a. The expression of KIR2DL4 on CD3+CD56+ cells before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $P > 0.05$.

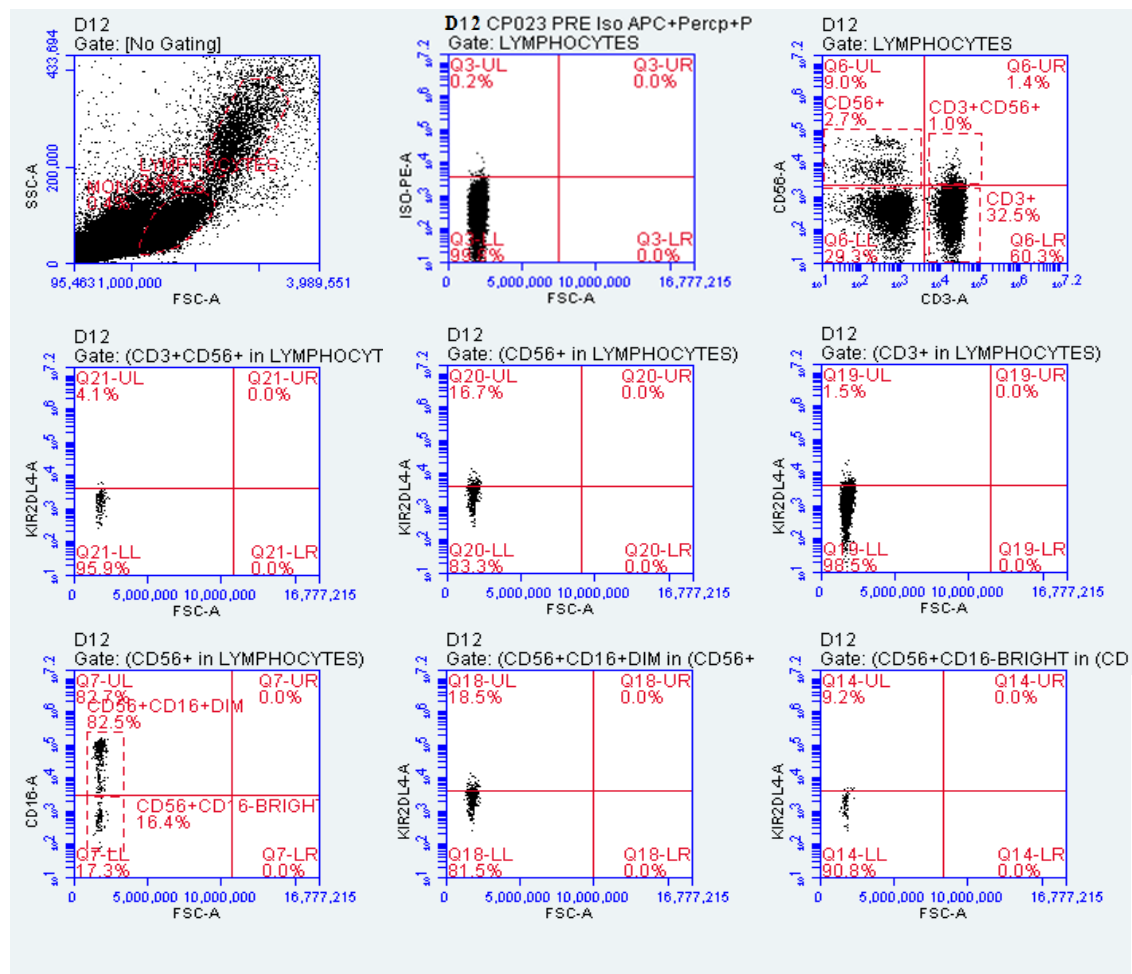


Fig. 7.15b. Acquisition plots for flow cytometry displaying the following order: Forward and side scatter, isotype control, CD3 and CD56 co-labelling, KIR2DL4 on CD3+CD56+, CD56+, CD3+, CD56^{dim} and CD56^{bright}.

7.19. Expression of KIR2DL4 by CD56+ cells pre and post renal transplantation

In the same way, the KIR2DL4 expressed by CD56+ cells was measured at sequential time points pre and post transplantation (Fig. 7.16) and there was slight increase in the proportion of cells expressing this protein after transplantation with the highest percentage recorded at 6, 2 and 4 months (11.40%, 10.03% and 9.20%) respectively compared to pre-transplant (7.54%). No statistically significant

difference was obtained after a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test was performed ($P= 0.2517$).

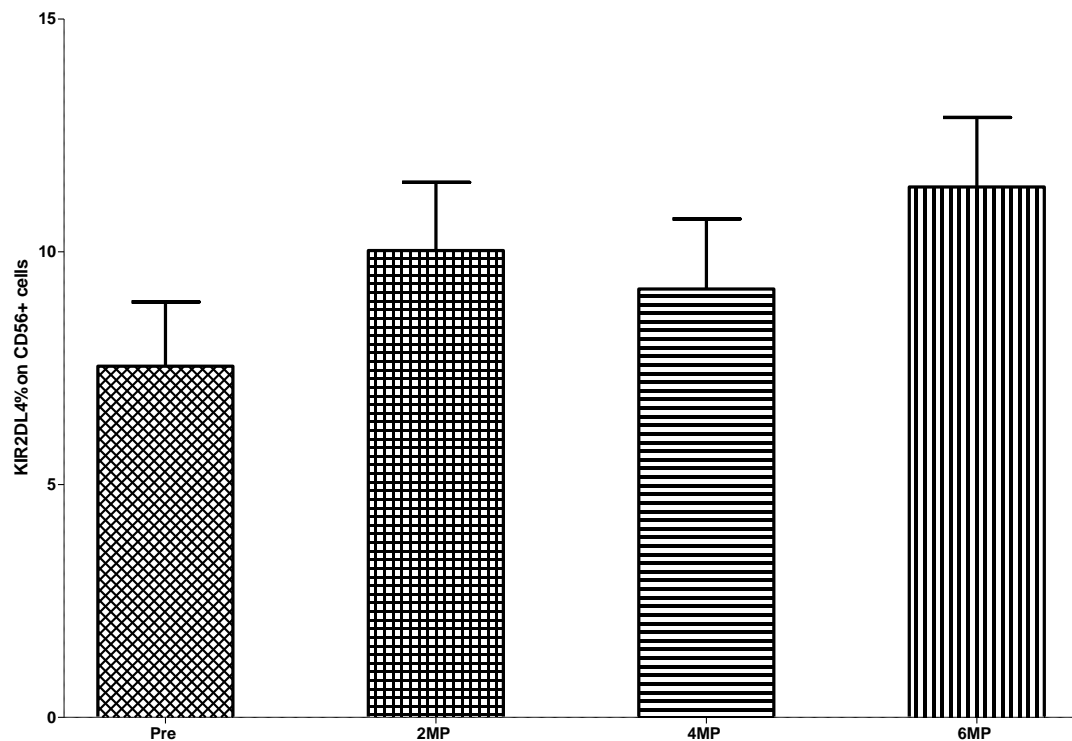


Fig. 7.16. The expression of KIR2DL4 on CD56+ cells before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $P>0.05$.

7.20. Expression of KIR2DL4 by CD3+ cells pre and post renal transplantation

Likewise, KIR2DL4 expression was assessed on CD3+ cells before and after transplantation (Fig. 7.17) and the highest proportion was recorded at 2 months post 11.95% followed by 6 months, 8.56% then at 4 months, 5.04% compared to pre-transplant (3.72%). No statistically significant difference was obtained after a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test was applied with ($P= 0.0832$).

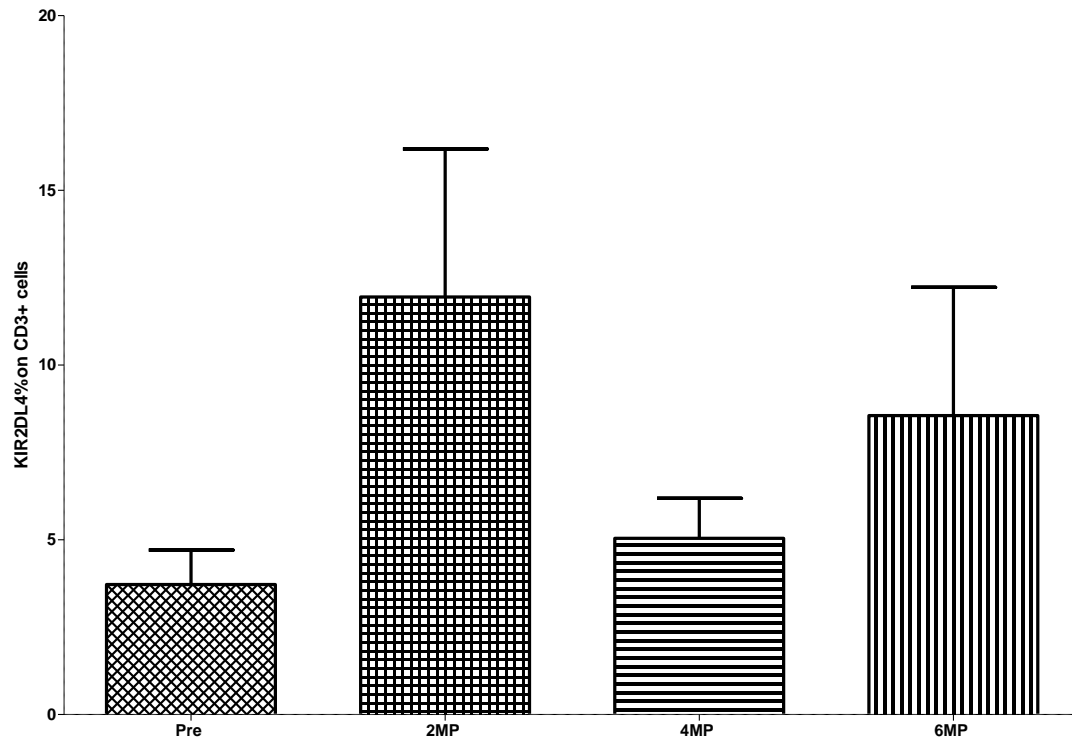


Fig. 7.17. The expression of KIR2DL4 on CD3+ cells before and after renal transplantation. Results are expressed as mean \pm SEM, $n = 13$, $P > 0.05$.

7.21. Expression of KIR2DL4 by CD16+CD56^{dim} cells pre and post renal transplantation

The same pattern was followed with CD16+CD56^{dim} cells. KIR2DL4 expression was assessed on these cells (Fig. 7.18) and the proportion of positive cells was increased following transplantation (pre=6.32%, 2 months=10.53%, 4 months=8.71% and 6 months=11.84%). When the groups were tested with a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test, no statistically significant difference was noted ($P=0.0844$). However, a significant difference was recorded between pre-and 6 months ($P<0.05$).

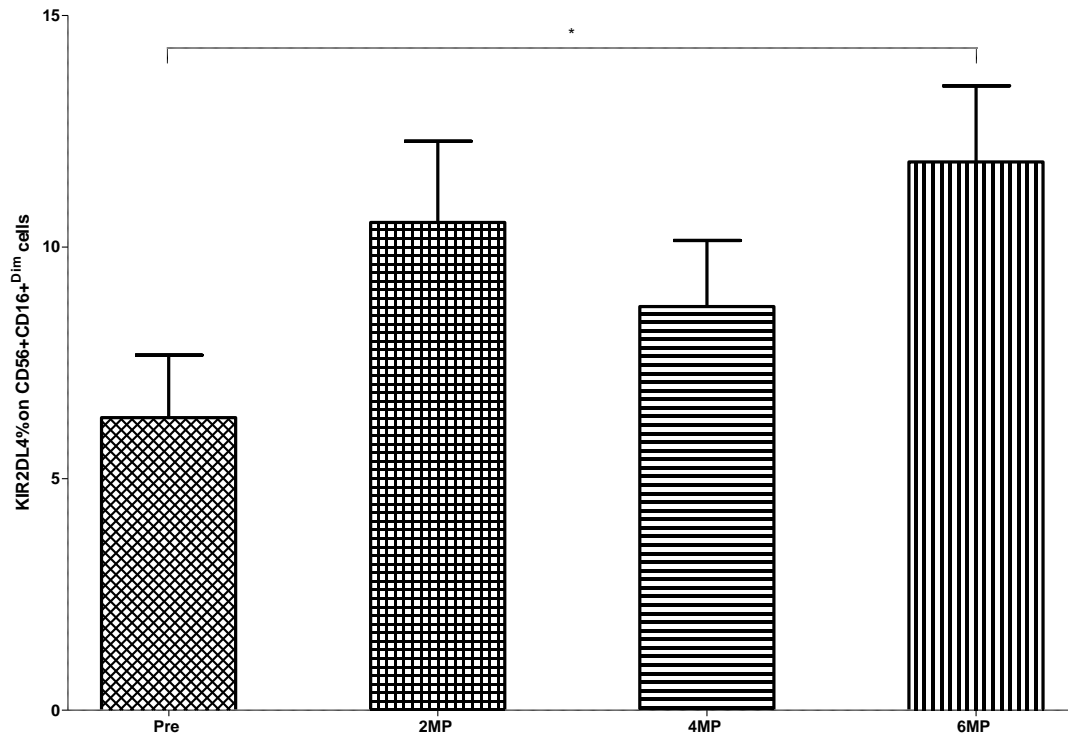


Fig. 7.18. The expression of KIR2DL4 on CD16+CD56^{dim} cells before and after renal transplantation. Results are expressed as mean \pm SEM, n = 13, * P<0.05.

7.22. Expression of KIR2DL4 by CD56^{bright}CD16⁻ cells pre and post renal transplantation

On CD56^{bright}CD16⁻ cells, the expression of KIR2DL4 was measured before and after transplantation (Fig. 7.19) and there was a slight increase in the proportion cells expressing this protein following transplantation (pre=9.39%, 2 months=11.54%, 4 months=11.11% and 6 months=12.04%) respectively. The group were tested with a Repeated Measures ANOVA, Dunnett's Multiple Comparison Test and no statistically significant difference was recorded with (P=0.7491).

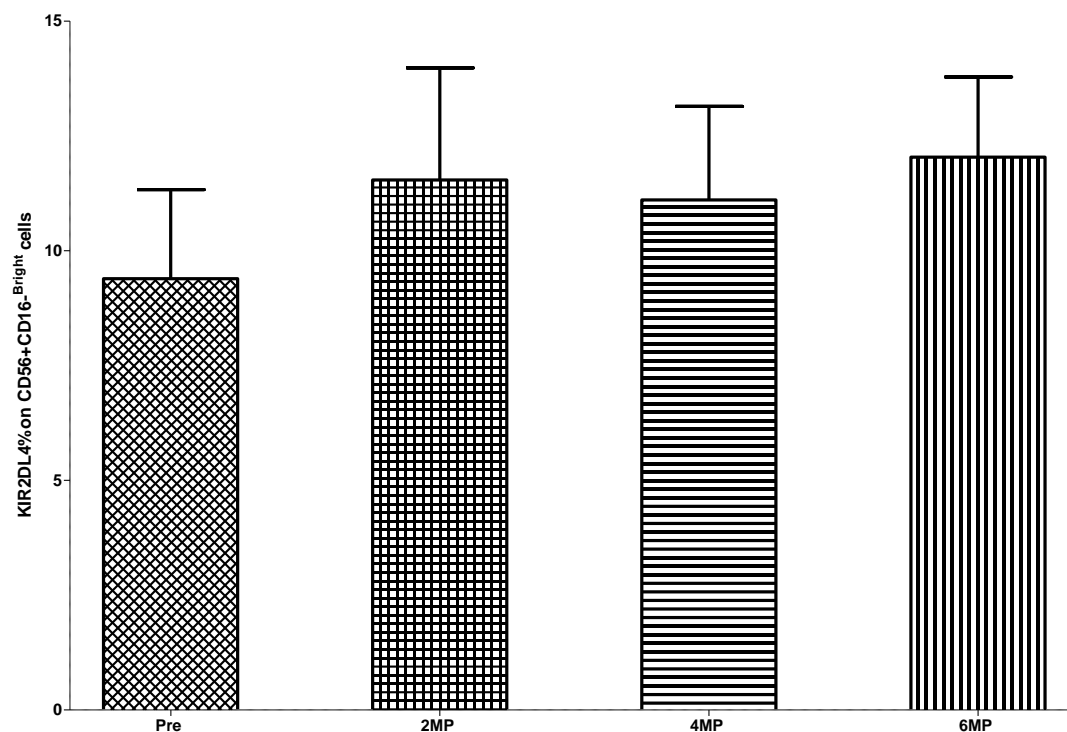


Fig. 7.19. The expression of KIR2DL4 on CD56^{bright} cells before and after renal transplantation. Results are expressed as mean \pm SEM, n = 13, P>0.05.

7.23. KIR2DL4 expression by various PBMC populations pre-and 2 months after transplantation

The expression of KIR2DL4 was evaluated on various PBMC populations before and 2 months after transplantation in 7 patients who were only able to provide two clinical samples. PBMCs were extracted and phenotyped with anti-KIR2DL4 mAbs and data were acquired with Accuri C6 flow cytometry. All the lymphocyte subsets showed patterns of upregulation of the protein after transplantation (Fig. 7.20), however, there were no statistically significant differences noted (P>0.05) when the groups were compared with a Paired t test (two-tailed; Table 7.9).

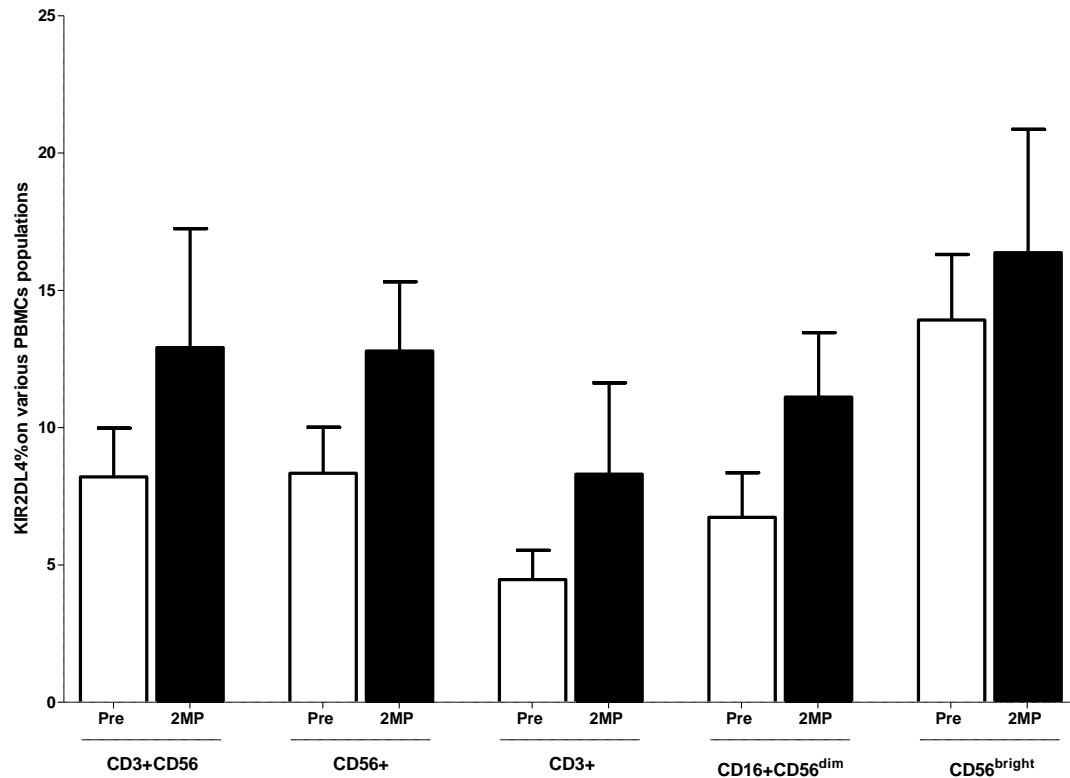


Fig. 7.20. The expression of KIR2DL4 on various PBMC populations before and 2 months after renal transplantation. Results are expressed as mean \pm SEM, n = 10, P>0.05.

Mean KIR2DL4% on various PBMC populations			
PBMCs	Pre-transplant	2 months post	P value
CD3+CD56	7.738	12.25	0.2175
CD56+	8.398	12.01	0.2903
CD3+	4.174	7.253	0.3264
CD16+CD56 ^{dim}	6.773	10.54	0.1898
CD56 ^{bright}	14.01	15.38	0.8032

Table 7.9. Paired t test comparing KIR2DL4 expression on various PBMC populations before and 2 months after transplantation, n=10, P>0.05

7.24. KIR2DL4 genotyping

KIR2DL4 genotyping was performed on DNA samples from renal transplant patients involved in the study to test whether the occurrence of 9A or 10A alleles in exon 6 of KIR2DL4 gene has any influence on KIR2DL4 expression on PBMCs (pre and 2 months post transplantation). DNA samples were sequenced to detect the presence of 9A and 10A in exon 6. The frequency of these alleles was recorded and KIR2DL4 expressed by CD3+CD56+, CD16+CD56^{dim} and CD56^{bright}CD16- cells were analyzed according to the presence of 9A and 10A alleles in homozygotes. The data were compared with an unpaired t test (two tails) and no statistically significant differences were obtained among all the groups, $P>0.05$, (Fig. 7.21 and Table 7.10). The proportions of KIR2DL4+ cells in cells from 10A subjects was higher in CD3+CD56+ and CD16+CD56^{dim} cells and similar proportions were seen in CD56^{bright}CD16- cells. Unlike pre-transplant expression, the 2 months post transplant samples showed increased proportions of KIR2DL4+ cells in all cell groups, especially in 9A subjects, where all cell types had higher proportions of KIR2DL4+ cells than the 10A subjects, however, no significant differences were obtained when an Unpaired t test (two tails) was performed ($P>0.05$; Fig. 7.21 and Table 7.10).

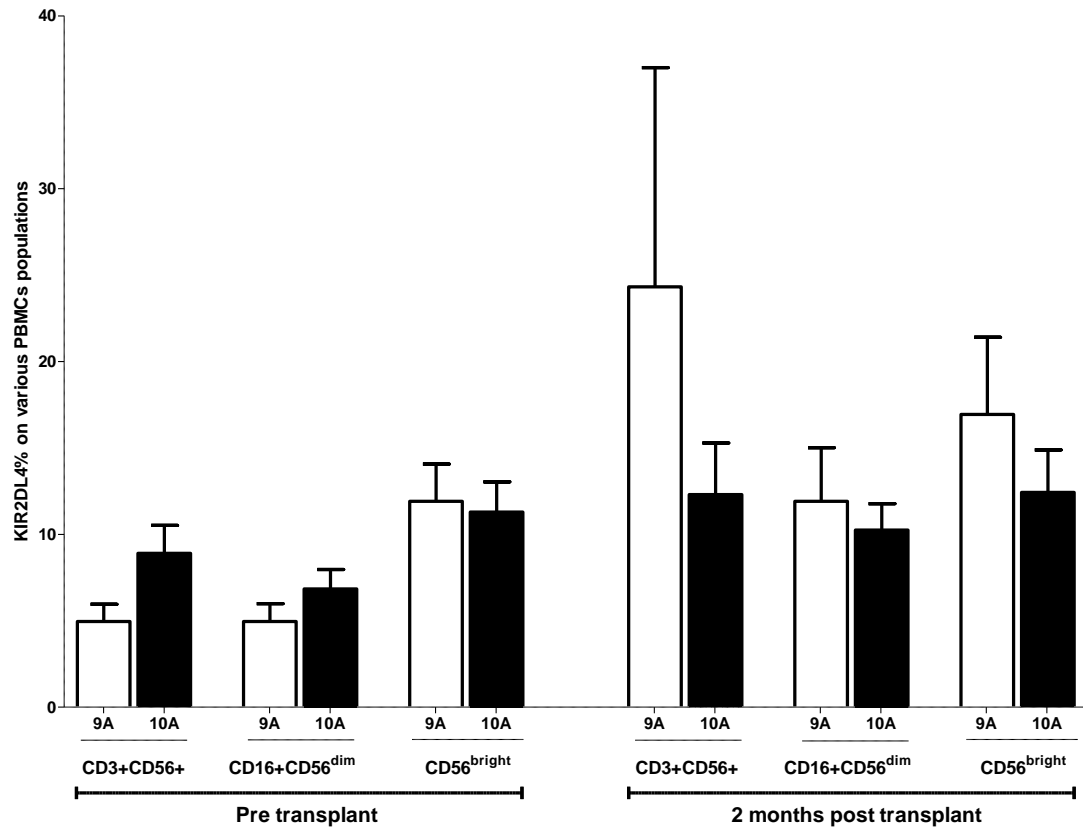


Fig. 7.21. The expression of KIR2DL4 on some PBMC groups according to the occurrence of 9A and 10A alleles in pre-transplant patient samples, (empty columns, 9A, n = 4) and (filled columns, 10A, n = 19). Results are expressed as mean \pm SEM, $P > 0.05$.

cells	9A	10A	P value
CD3+CD56+	4.960	8.902	0.2882
CD16+CD56 ^{dim}	4.958	6.842	0.4655
CD56 ^{bright}	11.92	11.29	0.8771
cells	9A	10A	P value
CD3+CD56+	24.33	12.30	0.1705
CD16+CD56 ^{dim}	11.91	10.25	0.6525
CD56 ^{bright}	16.94	12.43	0.4436

Table 7.10. The mean proportions of KIR2DL4 expressed by NK and non-NK cells in pre-transplant patient's samples and 2 months later. (9A, n = 4) and (10A, n = 19).

7.25. Summary and discussion of HLA-G and KIR2DL4 protein expression and their genotyping in renal transplant patients

To sum up, in renal transplant patients, the expression of HLA-G was significantly upregulated in CD3+CD4+CD45RO+ cells in pre vs 2 months, in addition to CD3+CD4+CD69+ lymphocytes in pre vs 2 months, CD14+HLA-DR+ cells in pre vs 6 months and CD19+ cells in pre vs 2 months. The remaining subsets have upregulated HLA-G but not to significant levels. While, the 14 bp genotyping and SNPs frequency have not shown any correlation with HAL-G levels. On the other hand, most KIR2DL4 expressing cells have not shown significant augment after transplantations with exception of CD56^{dim} cells which demonstrated significant increment of KIR2DL4 after 6 months of transplantation. Genotyping of 9A and 10A seen not to be influencing KIR2DL4 expression as all alleles variant had detectable amount of the protein Table 7.11.

Figure	Molecule	Cell population	CMV
7.2	HLA-G	CD3+CD4+CD45RO	Not applicable
7.5	HLA-G	CD3+CD4+CD69+	Not applicable
7.12	HLA-G	CD14+HLA-DR+	Not applicable
7.13	HLA-G	CD19+	Not applicable
7.18	KIR2DL4	CD56 ^{dim}	Not applicable

Table 7.11. Summary of significant differences in proportions of HLA-G and KIR2DL4 in renal transplant patients pre and post transplantation.

In renal transplant patients, the expression of HLA-G recorded slight elevation in most of the cells populations with significant increases observed in CD4+ lymphocytes, CD19+ cells and in monocytes following transplant procedure. This could be attributed to early activation of T and B cells and even monocytes after

transplantation due to tissue sensitization or mismatches of other HLA proteins rather than the main group of MHC used for tissue matching (Lu et al., 2011). The protocol applied in the transplantation involves the administration of Prograf or Mycophenolate Mofetil, which may directly or indirectly influence the expression of HLA-G through alteration or modulation of cytokines synthesis such as IL-4, IL-10 and IL-12 released by activated T lymphocytes (Daniel et al., 2005). So, this upregulation of HLA-G molecule on various PBMCs may indicate the effects of immunosuppressive medication on HLA-G or an ongoing tolerance mechanism which is not fully clear. This could favour tissue acceptance and delay any episodes of early rejection as well as suppressing those cells associated with initiation of tissue rejection (Lu et al., 2011). HLA-G exert its influence on effector immune cells by suppressing NK and CTL cells cytotoxic activities and/or by inhibiting alloproliferation of helper T cells (Carosella et al., 2003).

On the other hand, no significant correlation was recorded between the 14bp ins/del genotype, the SNPs and expression levels of HLA-G. Previous works have revealed such correlation and the data presented here could be attributed to the fact that genetic variation may be influenced by ethnic background as the group involved in the study were from various origins (Eduardo A. Donadi et al., 2011). Also, relatively small numbers of transplant patients were involved in the current study which may not reflect a powerful statistical sampling, a larger sample size may produce a different outcome.

Similarly, the SNPs incidence were not significantly associated with elevated levels of HLA-G and this could be due to various elements may influence the translation of HLA-G protein (Moreau, Flajollet, & Carosella, 2009) like the alterations in the SNPs occurring in the promoter region and the 3'UTR which may affect the levels of mRNA levels and hence the amount of protein displayed on cell surface or sHLA-G. In addition, the incidence of SNPs in intron 1-5 could exert regulatory functions affecting the splicing of the molecule (Donadi et al., 2011).

Regarding KIR2DL4 expression in transplant patients, this receptor revealed a trend toward increasing expression after renal transplantation on PBMCs surface, however, no significant expression was noted apart from on CD56^{dim} NK cells post transplantation. The detection of KIR2DL4 on various PBMCs sets could be due to the expression of HLA-G noted pre and post transplantation, this could signify the influence of HLA-G through the expression of its ligands such as KIR2DL4, ILT2 and

ILT4 particularly in NK cells and other T cells (Vera Rebmann et al., 2014). The implication behind KIR2DL4 expression could be seen as an inhibitory receptor that may induce tolerance to allograft and maintain the function of and prolong survival of renal graft (Rouas-Freiss, Naji, Durrbach, & Carosella, 2007).

On the other hand, the results of transplant patients have shown no direct influence of the 9A and 10A with KIR2DL4 expression on NK and other lymphocytes, as previously reported. This may be explained by the specificity of the mAb clone used in those previous work did not capture surface KIR2DL4 receptor which differ from the one applied in the current study experiments. Moreover, all patients in this study were receiving immunosuppressive drugs that may affect the receptor expression directly or indirectly through the upregulation of HLA-G could may in upregulate of its ligands including KIR2DL4 (Akhter et al., 2012; Rebmann et al., 2014).

CHAPTER EIGHT RESULTS

**Immunosuppressive drugs effects of HLA-G,
CD103, BAFF and BAFF-R expression**

In this chapter, the immunosuppressive drugs, Prograf (Tacrolimus) and Mycophenolate mofetil, used in routine renal transplantation pre or post-operative procedures were utilised to assess their effects on the expression of HLA-G, CD103, BAFF and BAFF-R. Having shown in Chapters 5 & 6 that CMV antigens can upregulate all of these molecules on some cell populations, the rationale was to test whether the immunosuppressive drugs routinely given to renal transplant patients had any additional effect on their expression, either alone or in combination with CMV antigens. These experiments involved the extraction of PBMCs from five healthy subjects, the mononuclear cells were stimulated with the physiological concentrations (Tacrolimus/Prograf= 10ng/ml and Mycophenolate=3µg/ml) that are regularly used *in vivo* in renal transplant patients. The drugs were incorporated in parallel with or without CMV particles in culture media in five different combinations (control non-stimulated PBMCs, CMV particles alone, CMV particles + Prograf, CMV particles + Mycophenolate, CMV particles + Prograf + Mycophenolate and Prograf + Mycophenolate alone). The cells were incubated for seven days and following culture the PBMCs were recovered and labelled with monoclonal Abs to assess the expression of the HLA-G, CD103, BAFF and BAFF-R antigens in addition to the basic markers for each cell subpopulation. The labelled cells were then acquired with Accuri C6 flow cytometry and data were analysed with the software provided.

8.1. The effects of Immunosuppressive drugs on HLA-G expression after culture with or without CMV particles

PBMCs extracted from healthy subjects were incubated for seven days with culture media that included media only, CMV particles, CMV particles with different combinations of Prograf and Mycophenolate in addition to media with Prograf and Mycophenolate alone without CMV particles. After culture, the mononuclear cells were labelled with various monoclonal Abs to assess the HLA-G expression on different populations of PBMCs.

8.1.1. Expression of HLA-G on CD3+CD4+CD69+ lymphocytes after treatment with immunosuppressive drugs

HLA-G expression on CD3+CD4+CD69+ cells was evaluated in response to different combinations of immunosuppressive drugs (Fig. 8.1), and there was fairly amount of HLA-G upregulation on these cells. Treatment with CMV particles + Prograf yielded the highest proportion of HLA-G+ cells in the CD3+CD4+CD69 population (11.28%) compared to 1.06% in the control. The second highest expression was noted in treatment with CMV particles + Prograf + Mycophenolate (9.626%) followed by treatment with CMV particles + Mycophenolate (4.742%), CMV particles (3.548%) and the least expression was recorded in Prograf + Mycophenolate treatment (0.72%). The groups of treatments were compared with the control with one-way ANOVA (Dunnett's Multiple Comparison Test) and there was a statistically significant difference overall ($P < 0.0001$). Particularly, significant differences were noted in (control vs CMV + Prograf, $P < 0.01$) treatment and comparison of control vs CMV particles + Prograf + Mycophenolate treatment ($P < 0.05$). No statistically significant differences were noted in the remaining combinations compared to the control group despite the elevated expression of HLA-G proportion displayed in these treatments. All the treatments have been shown to upregulate the expression of HLA-G apart from Prograf + Mycophenolate combination which has results similar or even lower than the control.

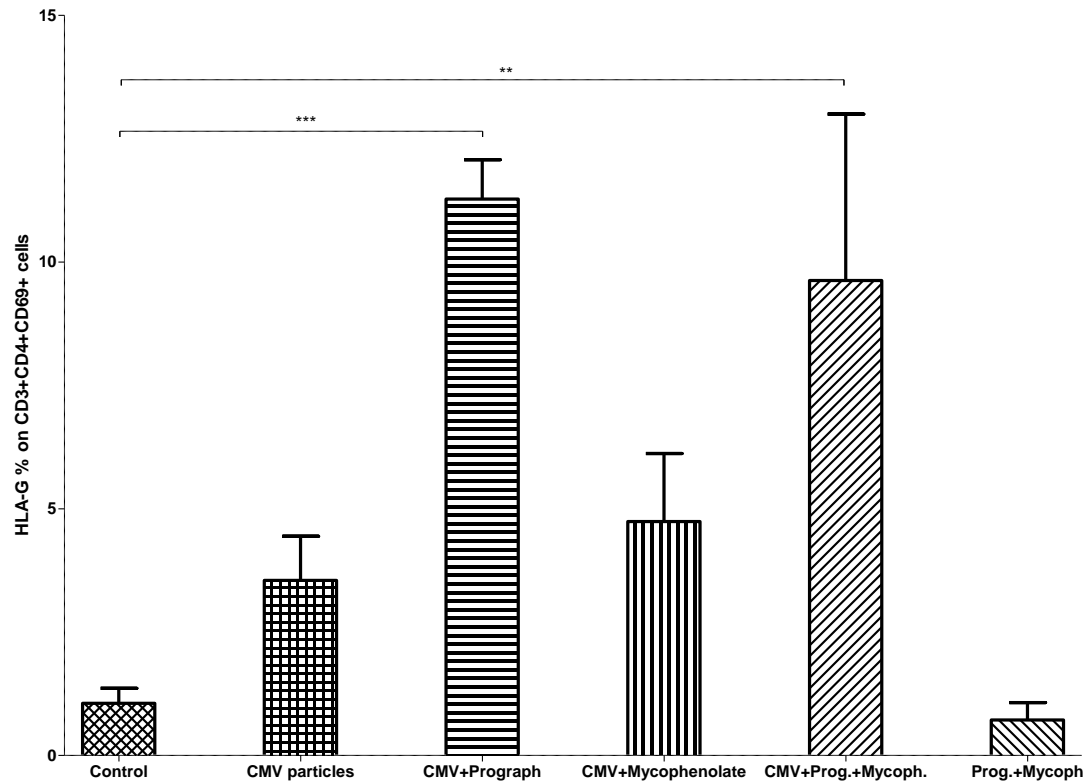


Fig. 8.1. The expression of HLA-G on CD3+CD4+CD69+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, *** $P < 0.01$. ** $P < 0.05$.

8.1.2. Expression of HLA-G by CD3+CD4+ lymphocytes after treatment with immunosuppressive drugs

The expression of HLA-G was assessed on CD3+CD4+ lymphocytes (Fig. 8.2) and the highest proportion was recorded in treatment with CMV particles + Mycophenolate (2.30%) vs 0.2% in the control. The remaining treatments influenced HLA-G expression in the following order: CMV particles 1.07%, CMV particles + Prograf 1.28%, CMV particles + Prograf + Mycophenolate 1.56% and Prograf + Mycophenolate 0.23%. When the groups were compared with the control utilising a one-way ANOVA (Dunnett's Multiple Comparison Test), there was statistically significant difference between the groups with overall $P = 0.0454$. In particular, there was a significant increase in CMV particles + Mycophenolate treatment vs control ($P < 0.05$).

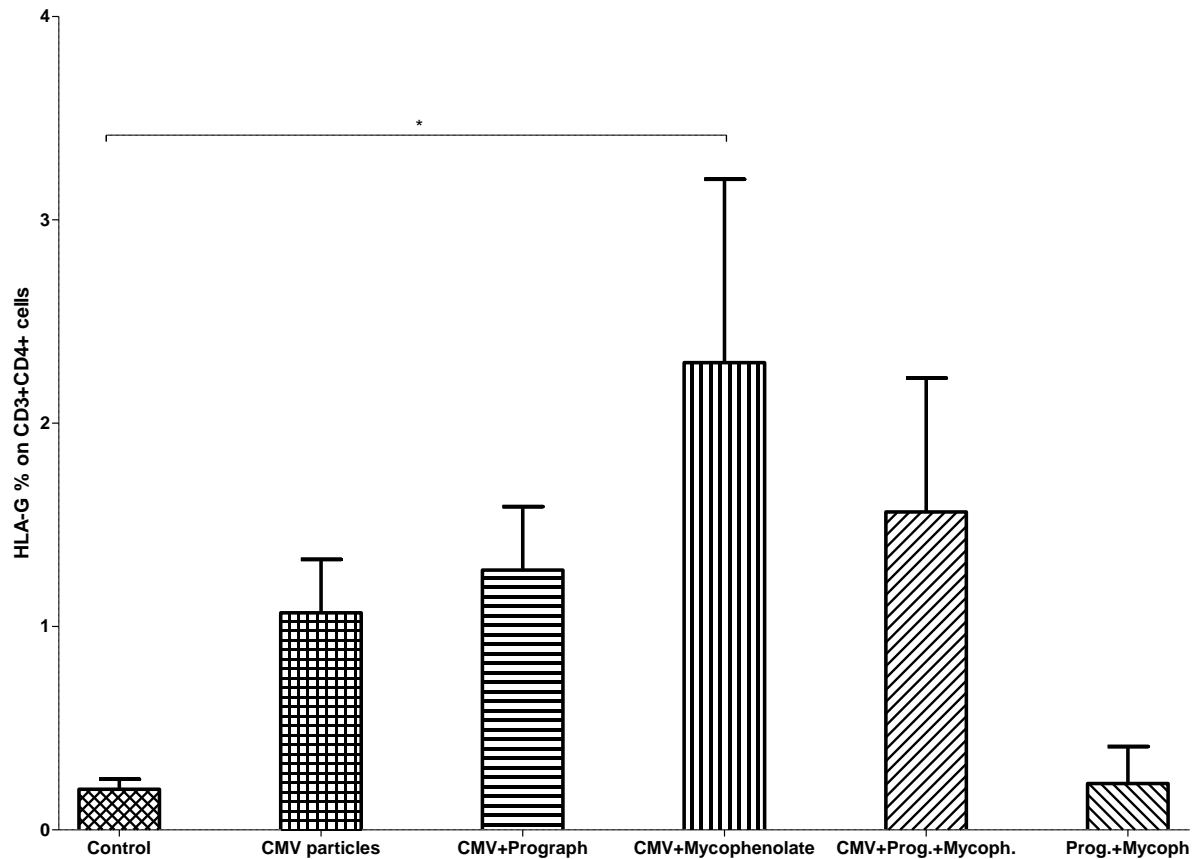


Fig. 8.2. The expression of HLA-G on CD3+CD4+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, * $P < 0.05$.

8.1.3. The expression of HLA-G on CD3+CD8+CD69+ cells after treatment with immunosuppressive drugs

Likewise, the HLA-G expression was assessed on CD3+CD8+CD69+ lymphocytes (Fig. 8.3) and there was a noticeably higher proportion of HLA-G+ cells in CMV particles + Mycophenolate (12.41%) treatment compared to 0.93% in the control group, While the lowest proportion of HLA-G expression was in Prograf + Mycophenolate treatment (0.70%). The proportion of HLA-G expression in PBMCs cultured with CMV particle was (3.42%), CMV particles + Prograf (3.6%) and CMV + Prograf + Mycophenolate (0.77%). The groups were then compared with the control using a one-way ANOVA (Dunnett's Multiple Comparison Test) and no statistically significant differences were observed ($P=0.3519$).

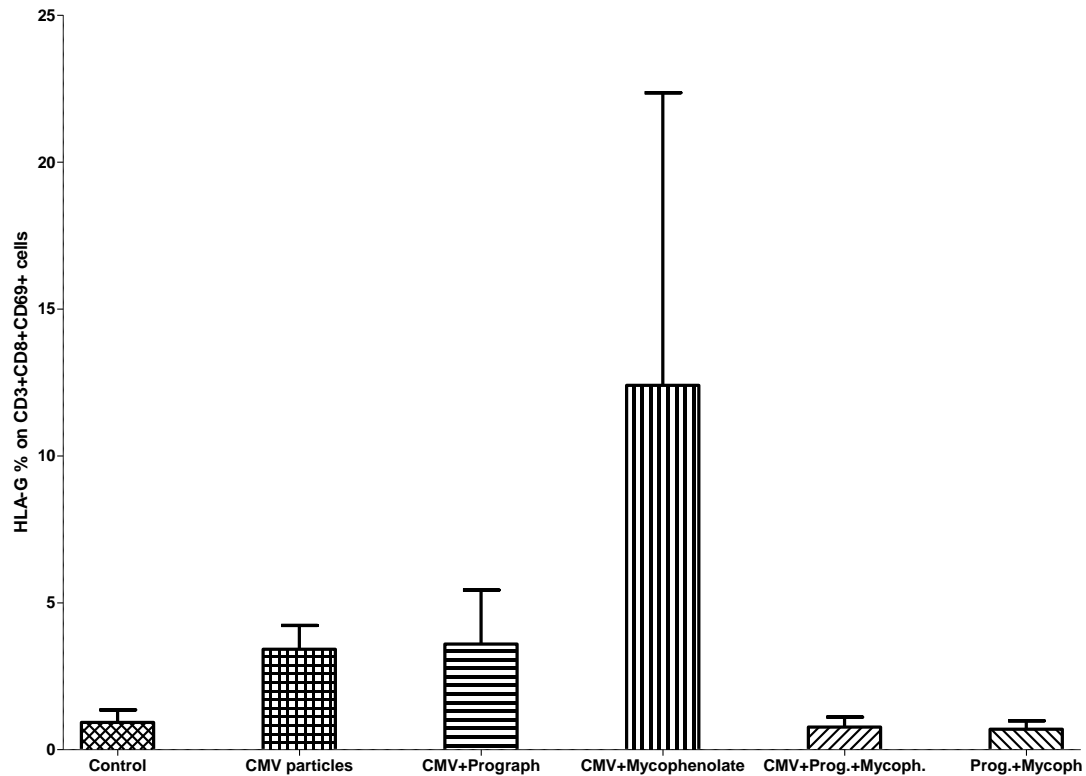


Fig. 8.3. The expression of HLA-G on CD3+CD8+CD69+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.1.4. HLA-G expression on CD3+CD8+ cells after treatment with immunosuppressive drugs

The proportion of HLA-G expression was also measured in CD3+CD8+ lymphocytes (Fig. 8.4) and as illustrated, CMV particles + Mycophenolate treatment showed the highest proportion of HLA-G induction (3.33%) vs 0.31% in the control. This was followed by CMV particles 1.88%, CMV particles + Prograf 1.14%, Prograf + Mycophenolate 1.02% and CMV + Prograf + Mycophenolate 0.56%. After comparing the groups with the control with a one-way ANOVA (Dunnett's Multiple Comparison Test), there were no statistically significant differences between them ($P = 0.1661$).

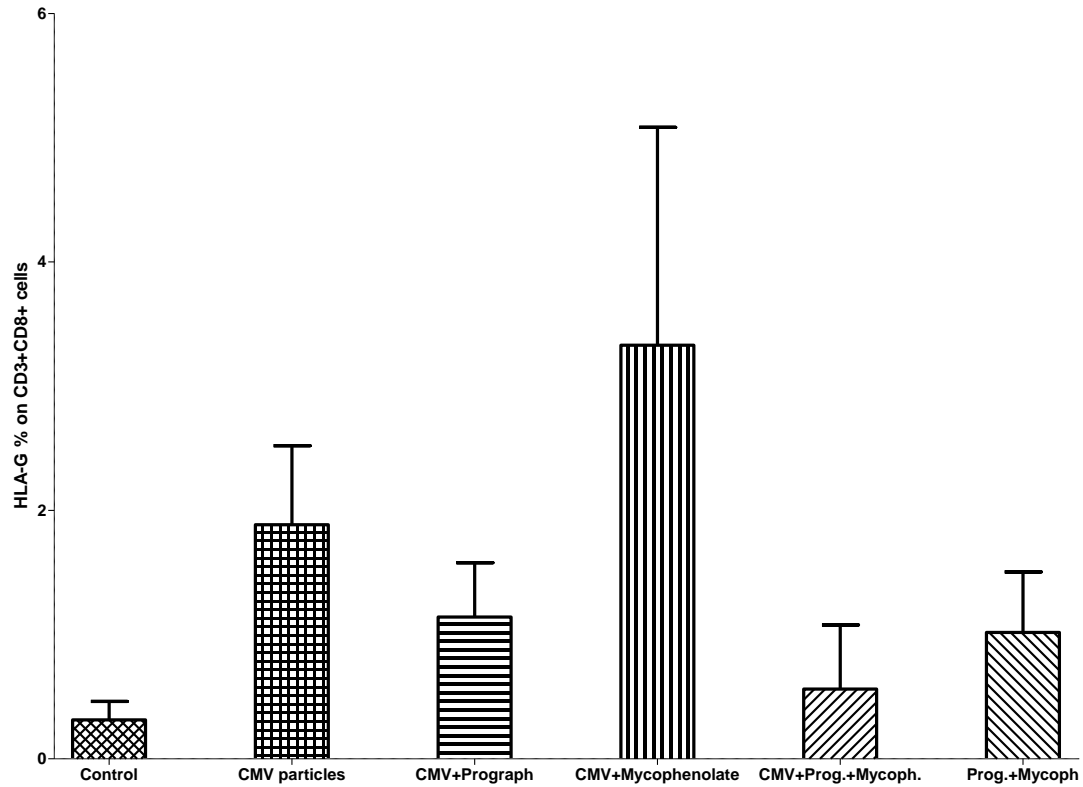


Fig. 8.4. The expression of HLA-G on CD3+CD8+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.1.5. HLA-G expression by CD19+ cells after treatment with immunosuppressive drugs

Upon assessing HLA-G expression on CD19+ cells (Fig. 8.5), CMV particle treatment had the greatest influence with a proportion of 14.51% positive cells, followed by CMV particles + Prograf (13.04%) vs 1.62% in the control. The remaining treatments induced the expression of HLA-G in the following order; CMV particles+ Mycophenolate 10.37%, CMV + Prograf + Mycophenolate 8.11% and Prograf + Mycophenolate 6.32%. When the groups were compared with a one-way ANOVA (Dunnett's Multiple Comparison Test) there was a statistically significant difference between the groups and the control ($P = 0.0244$). In particular there were significant differences in CMV particles vs control ($P < 0.05$) and CMV + Prograf vs control ($P < 0.05$).

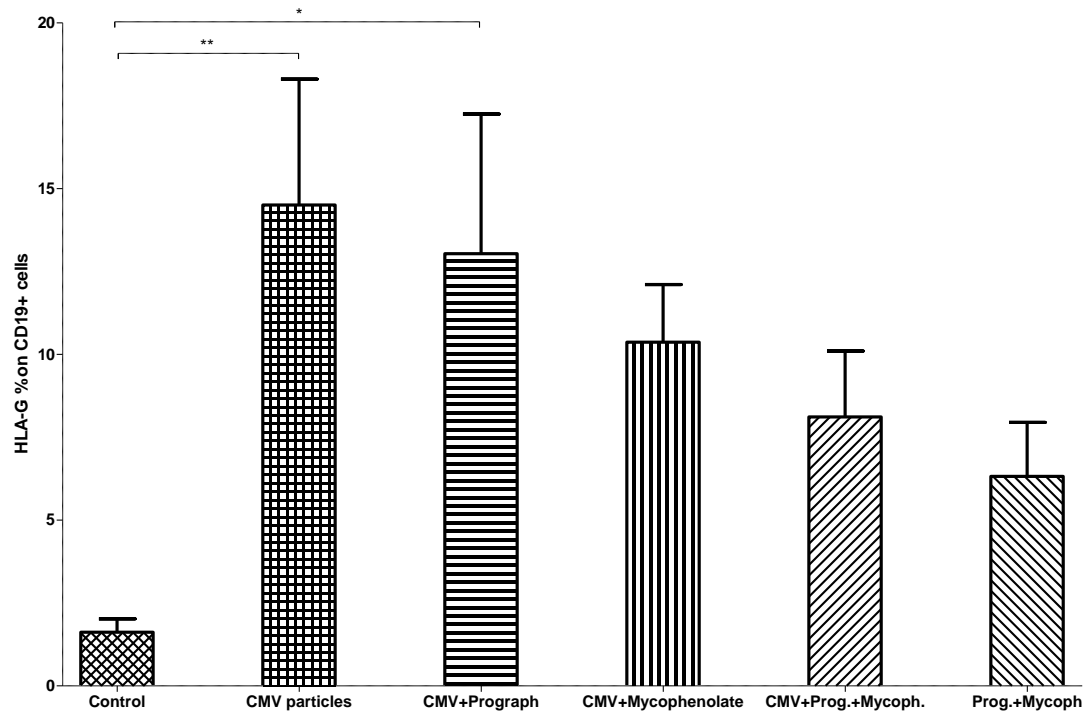


Fig. 8.5. The expression of HLA-G on CD19+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, * $P < 0.05$. ** $P < 0.01$.

8.1.6. Expression of HLA-G on CD14+HLA-DR+ cells after treatment with immunosuppressive drugs

Similarly, HLA-G expression on CD14+HLA-DR+ cells was measured following the induction with immunosuppressive drugs (Fig. 8.6). Clearly, CMV particle treatment upregulated HLA-G expression the most with a proportion of 23.91% positive cells vs 0.88% in the control. The second highest proportion was in treatment with CMV particles + Mycophenolate 15.72% followed by CMV particles + Prograf 7.83%, CMV + Prograf + Mycophenolate 4.33% and Prograf + Mycophenolate 4.19%. Upon performing a One-way ANOVA (Dunnett's Multiple Comparison Test) comparison with control, there was a statistically significant difference among them with overall $P = 0.0319$. Specifically, there was a significant difference in the control vs CMV particle treatment ($P < 0.05$).

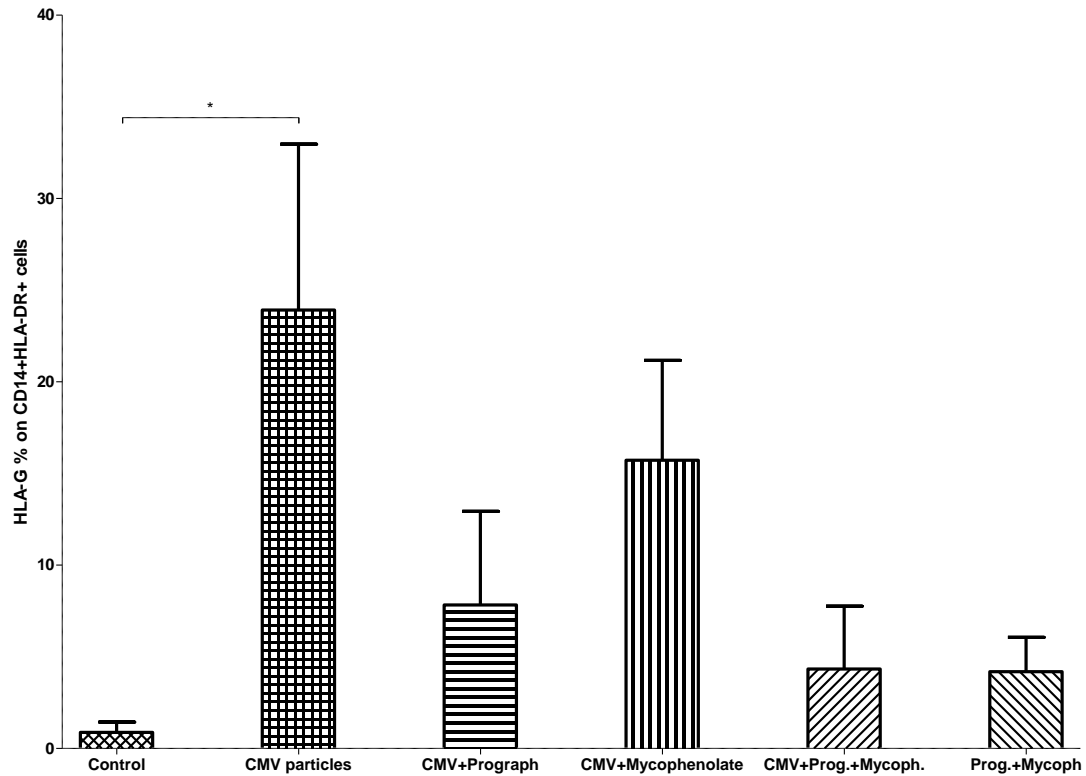


Fig. 8.6. The expression of HLA-G on CD14+HLA-DR+ cells following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, * $P < 0.05$.

8.1.7. HLA-G expression by CD3+CD56+ cells after treatment with immunosuppressive drugs

In the same pattern, HLA-G expression was evaluated on CD3+CD56+ cells (Fig. 8.7), as illustrated in the graph, high proportions of HLA-G+ cells were found with CMV particles + Prograf 31.58%, CMV particle 24.96% and CMV particles + Mycophenolate 19.40% treatments. This was followed by CMV + Prograf + Mycophenolate 13.73% and Prograf + Mycophenolate 12.86% vs 3.41% in the control. There was a statistically significant difference overall ($P = 0.0001$) when the groups were compared with the control using a one-way ANOVA (Dunnett's Multiple Comparison Test). Specifically, there were significant differences in CMV particles vs control ($P < 0.01$), CMV particles + Prograf vs control ($P < 0.01$) and CMV particles + Mycophenolate vs control ($P < 0.05$).

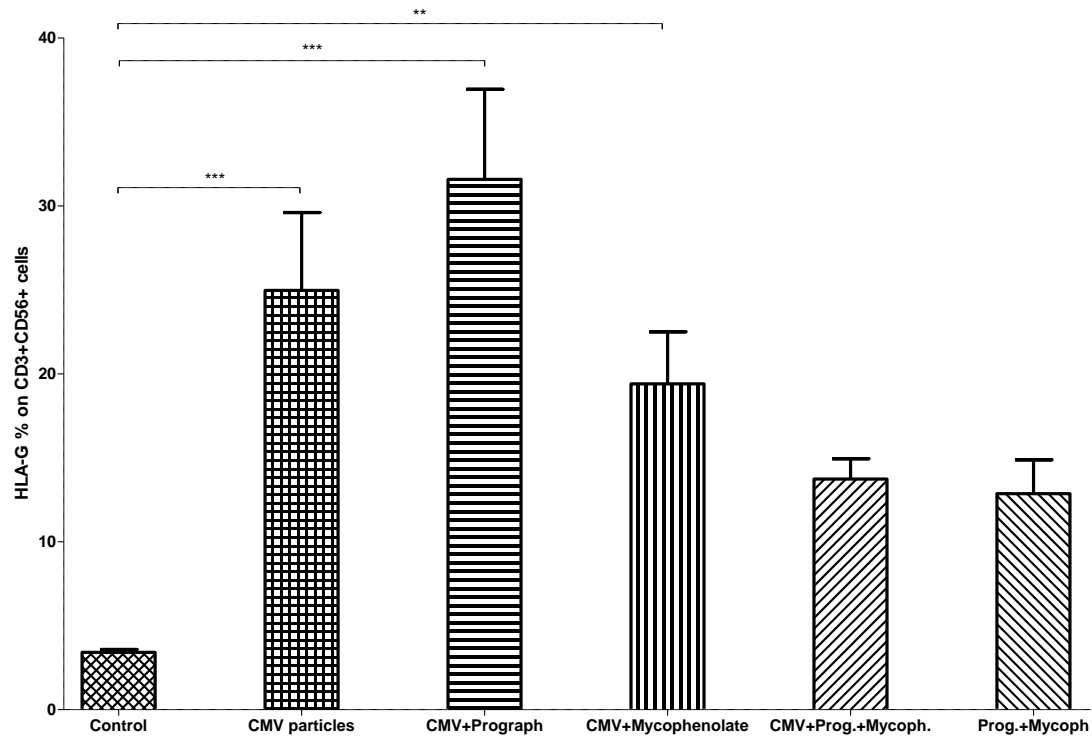


Fig. 8.7. The expression of HLA-G on CD3+CD56+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean +/- SEM, n = 5, ***P<0.01, **P<0.05.

8.1.8. HLA-G expression by CD56+ cells after treatment with immunosuppressive drugs

Finally, for the CD56+ cell group, HLA-G expression was also assessed in response to immunosuppressive drug treatments (Fig. 8.8). Clearly, CMV particles + Prograf treatment induced the highest proportion of HLA-G+ cells, 18.27% vs 0.73% in the control, followed by descending proportions in treatments with: CMV particles + Mycophenolate 12.55%, CMV particles 9.86%, CMV + Prograf + Mycophenolate 8.64% and Prograf + Mycophenolate 6.81%. After comparing the groups with the control utilizing a one-way ANOVA (Dunnett's Multiple Comparison Test) there was no statistically significant difference overall (P=0.0923) and only CMV particles + Prograf treatment vs control showed a significant difference (P<0.05).

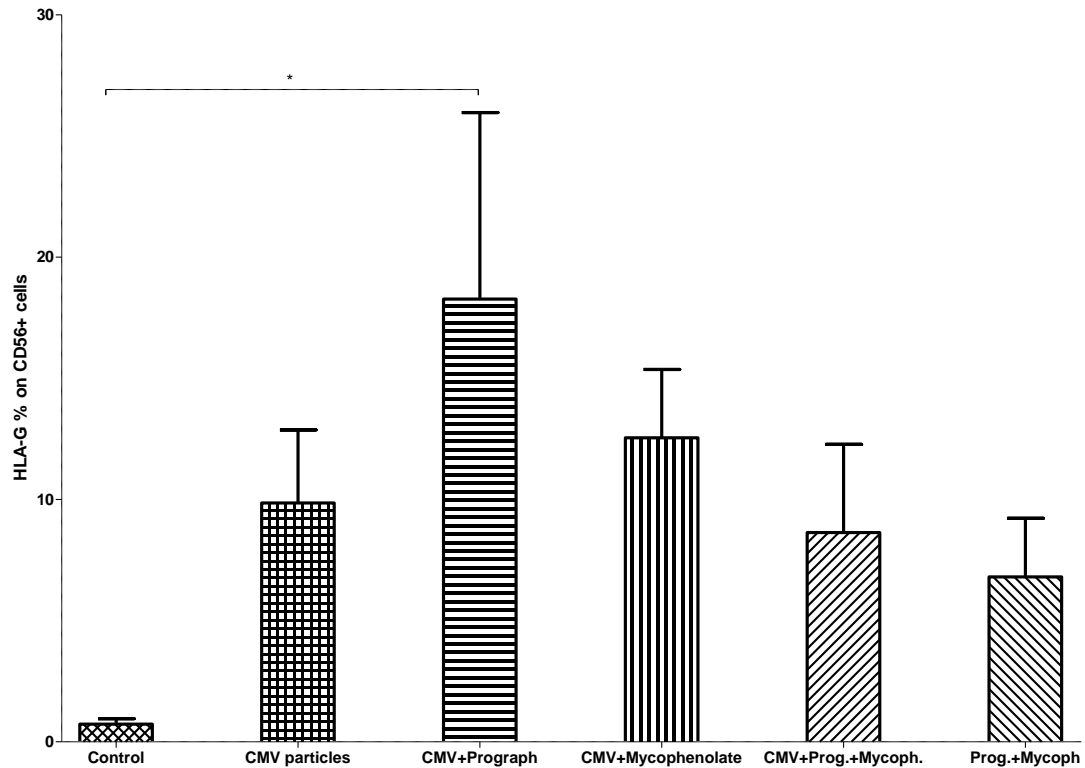


Fig. 8.8. The expression of HLA-G on CD56+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $*P < 0.05$.

8.2. Effect of Immunosuppressive drugs on CD103 expression in cultured PBMCs

Parallel to HLA-G assessment, CD103 expression was studied on various PBMC populations after culture with immunosuppressive drugs (Prograf and Mycophenolate) and CMV particles in the same combinations mentioned earlier. After culture, the PBMCs were recovered and phenotyped with monoclonal Abs specific for CD103 and HLA-G in addition to the basic subpopulation marker for each cell groups. Data obtained from Accuri C6 flow cytometry were analysed with the software provided.

8.2.1. The effects of in vitro Immunosuppressive drugs on CD103 expression by CD3+CD4+ cells

The expression of CD103 was studied on CD3+CD4+ cells following culture with immunosuppressive medications (Fig. 8.9). Clearly, the highest proportions of CD103+ cells were expressed in CMV particles + Prograf 4.90% and CMV particles + Mycophenolate 4.49% vs 0.06% in the control, whereas the remaining treatments showed relatively low induction of CD103 after culture (CMV + Prograf + Mycophenolate 1.664%, CMV particles 1.606% and Prograf + Mycophenolate 0.16%). Upon performing a one-way ANOVA (Dunnett's Multiple Comparison Test), no statistically significant difference was noted ($P=0.5131$).

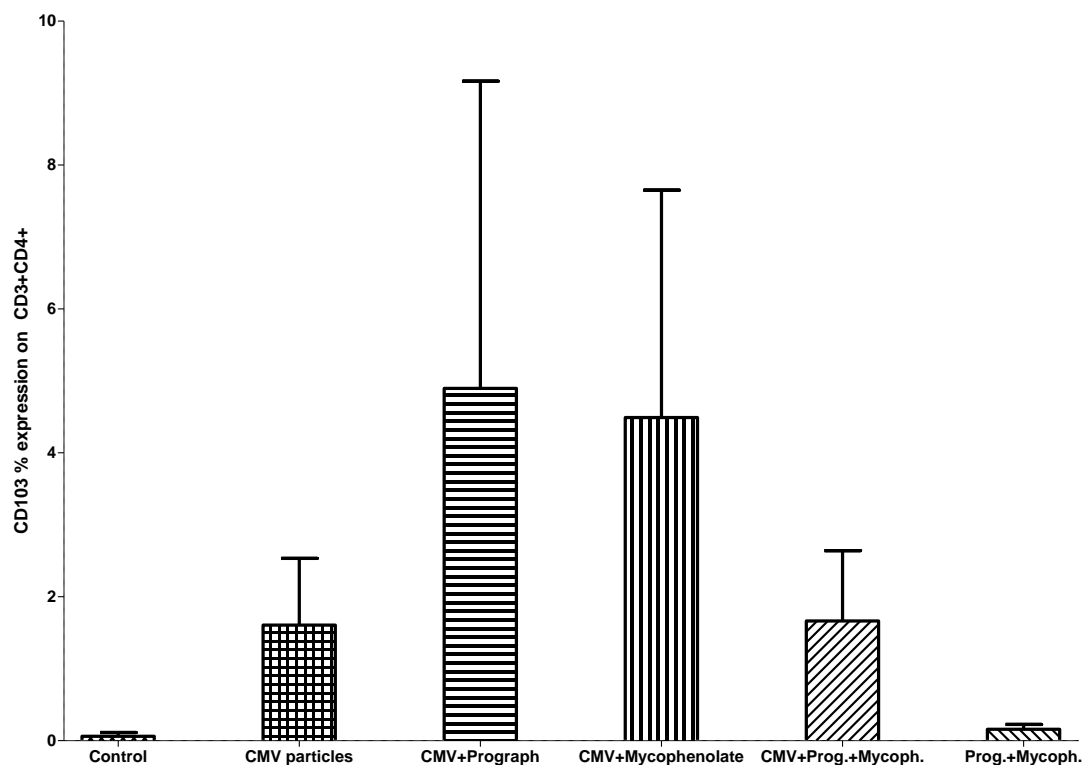


Fig. 8.9. The expression of CD103 on CD3+CD4+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P>0.05$.

8.2.2. The effects of Immunosuppressive drugs on CD103 expression by CD3+CD8+ lymphocytes

Likewise, on CD3+CD8+ lymphocytes, the expression of CD103 was assessed and as demonstrated (Fig. 8.10), the highest proportion of CD103 expression was recorded in CMV particles + Prograf 11.77% and CMV particles + Mycophenolate 9.08% vs 1.03% in the control. The rest of the treatments also upregulated the expression of CD103 after cell culture (CMV particles 6.746%, CMV + Prograf + Mycophenolate 3.16%) but not in Prograf + Mycophenolate treatment (0.71%). When the groups were compared with a one-way ANOVA (Dunnett's Multiple Comparison Test), no statistically significant difference was recorded ($P=0.1314$) despite the upregulation patterns in most of the drug treatment groups.

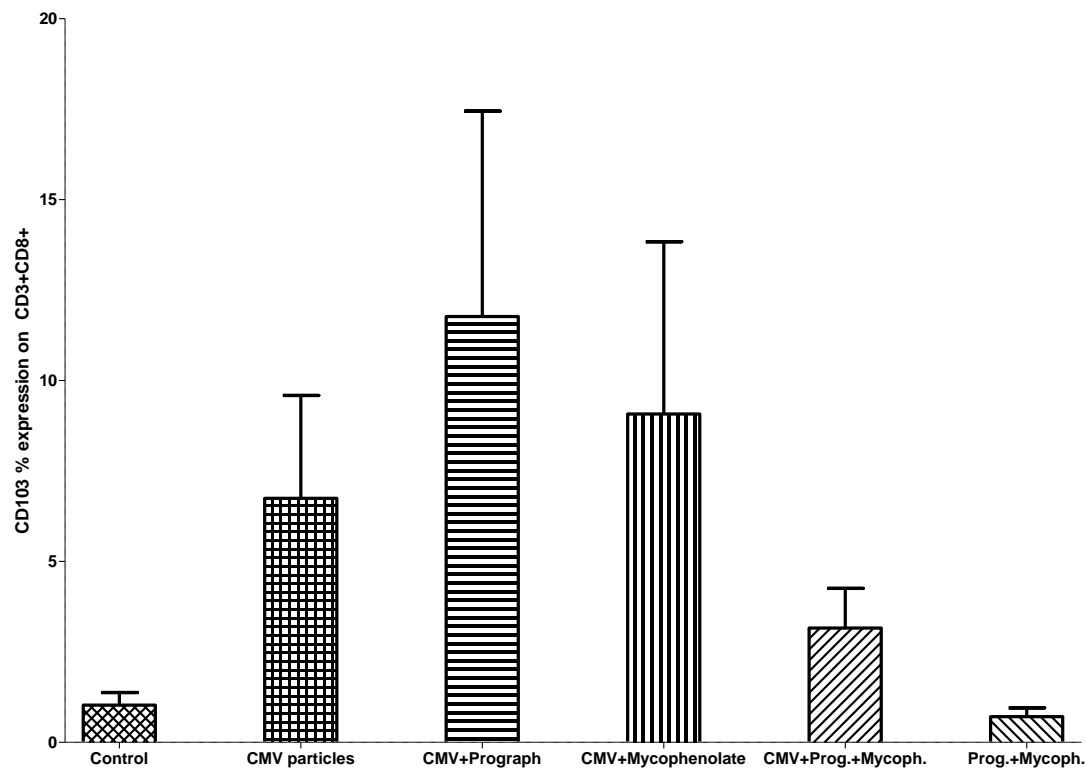


Fig. 8.10. The expression of CD103 on CD3+CD8+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P>0.05$.

8.2.3. The effects of Immunosuppressive drugs on CD103 expression by CD19+ cells

The effect of immunosuppressive drugs on CD19+ cells was assessed to study CD103 expression after culture with different combinations of the drugs (Fig. 8.11). Apparently, the proportion of CD103+ cells was increased in treatments with CMV particles 14.99%, CMV particles + Prograf 14.52%, CMV + Prograf + Mycophenolate 11.73% and CMV particles + Mycophenolate 9.65% in comparison to the control (1.28%), while a low proportion (3.45%) was expressed in Prograf + Mycophenolate treatment. After comparing the groups with the control using a one-way ANOVA (Dunnett's Multiple Comparison Test), no statistically significant difference was noted $P > 0.05$.

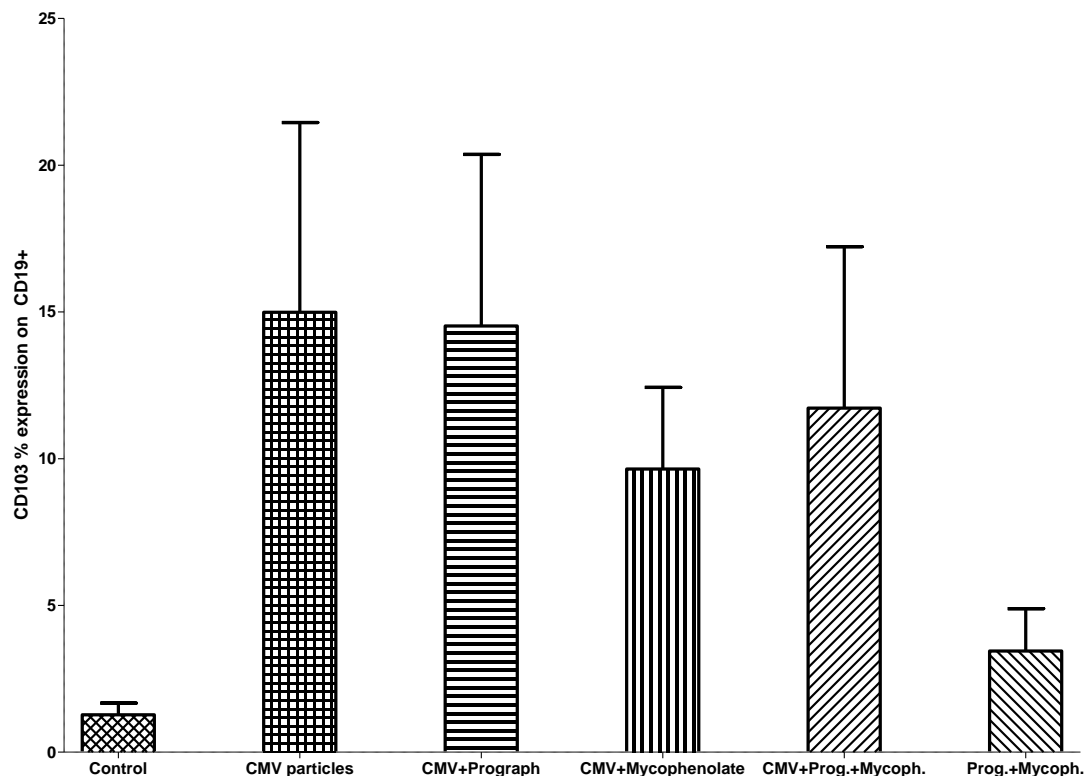


Fig. 8.11. The expression of CD103 on CD19+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.2.4. The effects of Immunosuppressive drugs on CD103 expression by CD3+CD56+ cells

In the same way, CD103 expression was evaluated on CD3+CD56+ lymphocytes (Fig. 8.12) and as depicted, the proportion of CD103+ cells in response to immunosuppressive treatment was highest in CMV particles + Prograf 30.02%, CMV particles + Mycophenolate 29.87% and CMV particles 26.40% vs 2.55% in control. This was followed by CMV + Prograf + Mycophenolate 18.85% and Prograf + Mycophenolate 6.53%. The groups were compared with the control utilizing a one-way ANOVA (Dunnett's Multiple Comparison Test) and there was no statistically significant difference ($P=0.1732$).

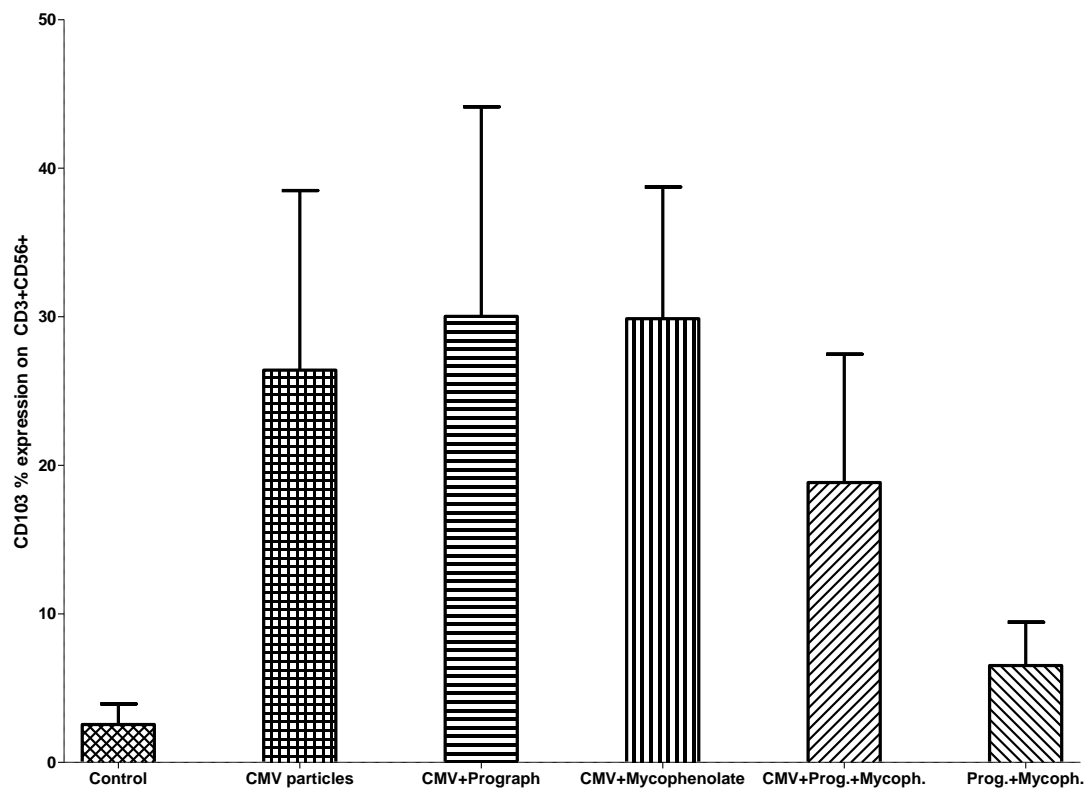


Fig. 8.12. The expression of CD103 on CD3+CD56+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P>0.05$.

8.2.5. The effects of Immunosuppressive drugs on CD103 expression by CD56+ cells

Upon studying CD103 expression on CD56+ cells in response to immunosuppressive drugs treatment (Fig. 8.13), there was clearly upregulation of this antigen in the following treatments: CMV particles + Prograf 35.35%, CMV + Prograf + Mycophenolate 30.42%, CMV particles + Mycophenolate 20.97%, Prograf + Mycophenolate 18.31% and CMV particles 11.20% vs 2.588 in the control. The groups were tested against the control with a one-way ANOVA (Dunnett's Multiple Comparison Test) and the overall P value was close to significance ($P=0.0745$). In particular, a significant difference was noted in CMV particles + Prograf vs control ($P<0.05$).

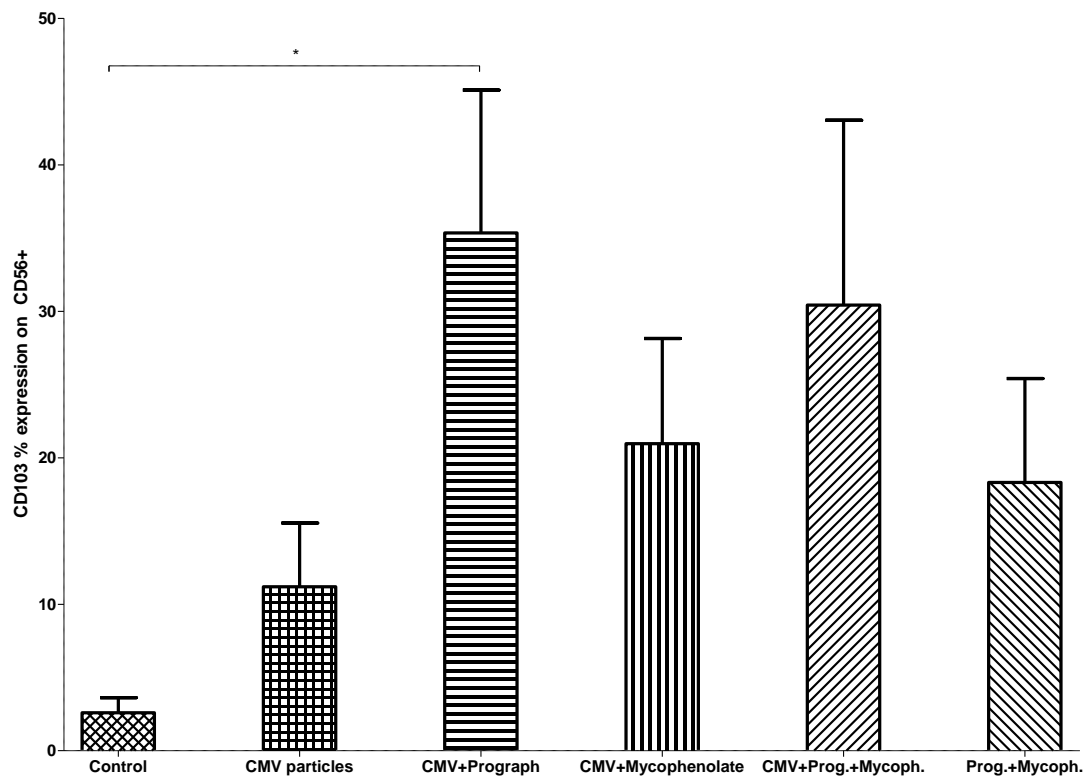


Fig. 8.13. The expression of CD103 on CD56+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $*P<0.05$.

8.3. Expression of HLA-G on CD103+ PBMCs after immunosuppressive drug treatment

The cells that were phenotyped with anti-CD103 mAbs were also assessed for expression of HLA-G in parallel with CD103 in response to immunosuppressive drug treatments; the same combinations of the medications were incorporated in the culture media and the PBMCs were incubated for 7 days. The PBMCs were recovered and labelled with monoclonal antibodies specific for HLA-G in addition to CD103 and the other subpopulation markers. The cells were gated for CD103, HLA-G was measured on various mononuclear cells and data were analysed with Accuri C6 software.

8.3.1. Expression of HLA-G on CD3+CD4+CD103+ cells following immunosuppressive drug treatment

The HLA-G expression was assessed in those PBMCs that were gated for CD3+CD4+CD103+ cells following culture with immunosuppressive drug combinations (Fig. 8.14). As it is clearly shown below, the following descending proportions of HLA-G were recorded after culture in the following order: Prograf + Mycophenolate 10.89%, CMV particles 8.42%, CMV particles + Prograf 7.32%, CMV particles + Mycophenolate 6.78% and CMV + Prograf + Mycophenolate 3.51% treatments. After applying a one-way ANOVA (Dunnett's Multiple Comparison Test), no statistically significant difference was noted ($P=0.6399$).

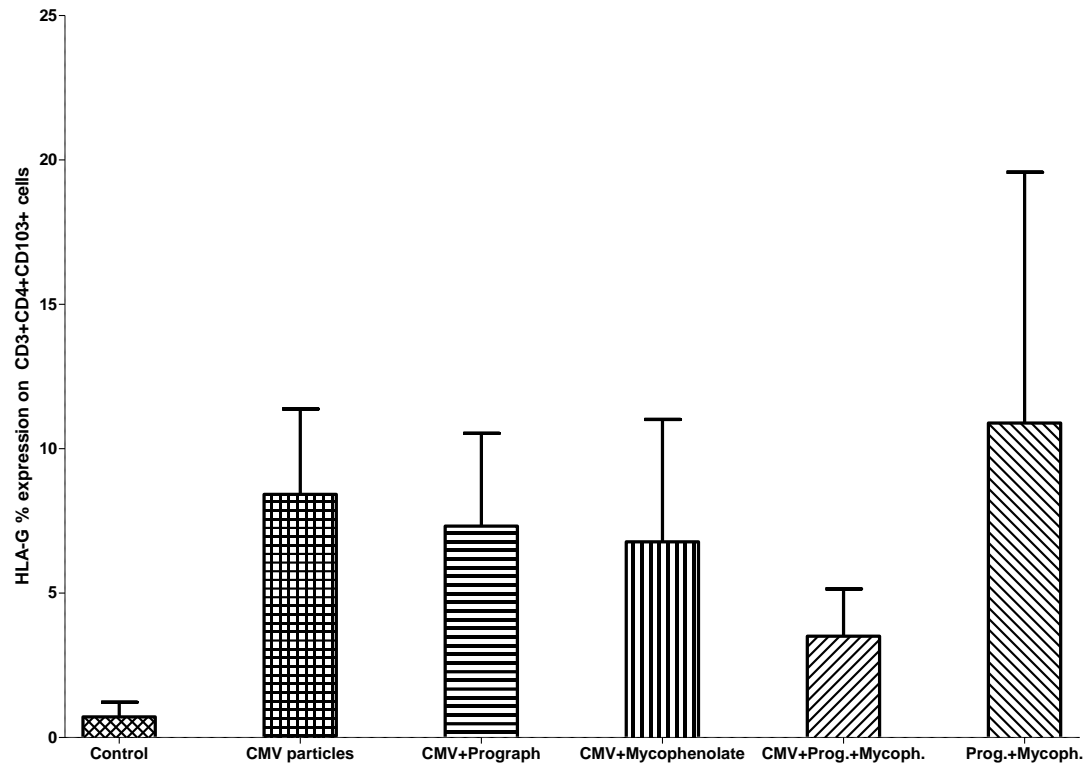


Fig. 8.14. The expression of HLA-G on CD3+CD4+CD103+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.3.2. Expression of HLA-G on CD3+CD8+CD103+ cells following immunosuppressive drug treatment

HLA-G was also assessed on the surface of CD3+CD8+CD103+ cells following culture with immunosuppressive medications (Fig. 8.15). The proportion of HLA-G expressed on cells treated with CMV particles + Prograf 5.65% and Prograf + Mycophenolate 4.20% was the highest among all the treatments. The proportions of HLA-G in the remaining treatments were as follow: CMV + Prograf + Mycophenolate 3.31%, CMV particles 2.35% and CMV particles + Mycophenolate 2.33%, while in the control it was 1.46%. The groups were compared with the control via one-way ANOVA (Dunnett's Multiple Comparison Test) and the test revealed no statistically significant difference ($P=0.8385$).

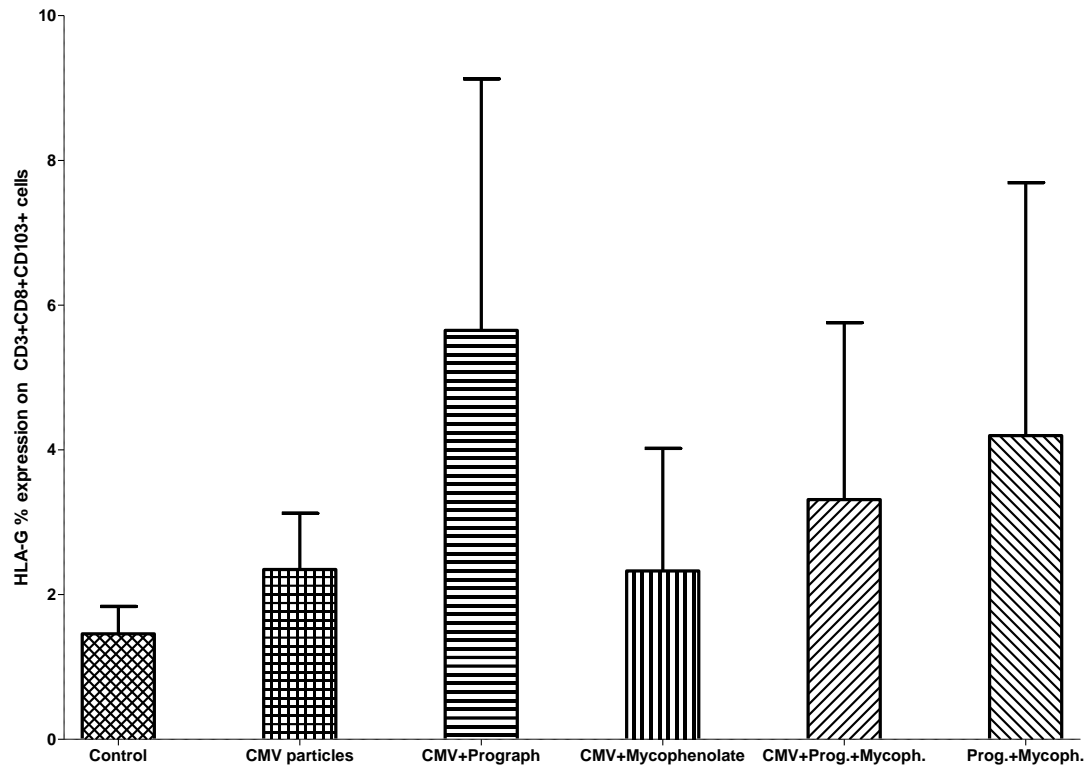


Fig. 8.15. The expression of HLA-G on CD3+CD8+CD103+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.3.3. Expression of HLA-G on CD19+CD103+ cells following immunosuppressive drug treatment

PBMCs positive for CD19 and CD103 markers were examined to evaluate the expression of HLA-G (Fig. 8.16) and as it is obvious in the graph below that most of the treatments upregulated the expression of HLA-G on CD19+CD103+ cells with the highest proportions in treatments with CMV particles + Prograf 29.18%, CMV particles 26.40%, CMV particles + Mycophenolate 23.94%, Prograf + Mycophenolate 23.65% and CMV + Prograf + Mycophenolate 16.11%, whereas in the control the proportion of HLA-G+ cells was 1.35%. The various treatments were compared with control using one-way ANOVA (Dunnett's Multiple Comparison Test) and there was a statistically significant difference with overall ($P = 0.0257$), in particular, statistically significant differences were noted between CMV particles + Prograf vs control ($P < 0.01$), CMV particles vs control ($P < 0.05$), CMV particles +

Mycophenolate vs control ($P<0.05$) and Prograf + Mycophenolate vs control ($P<0.05$).

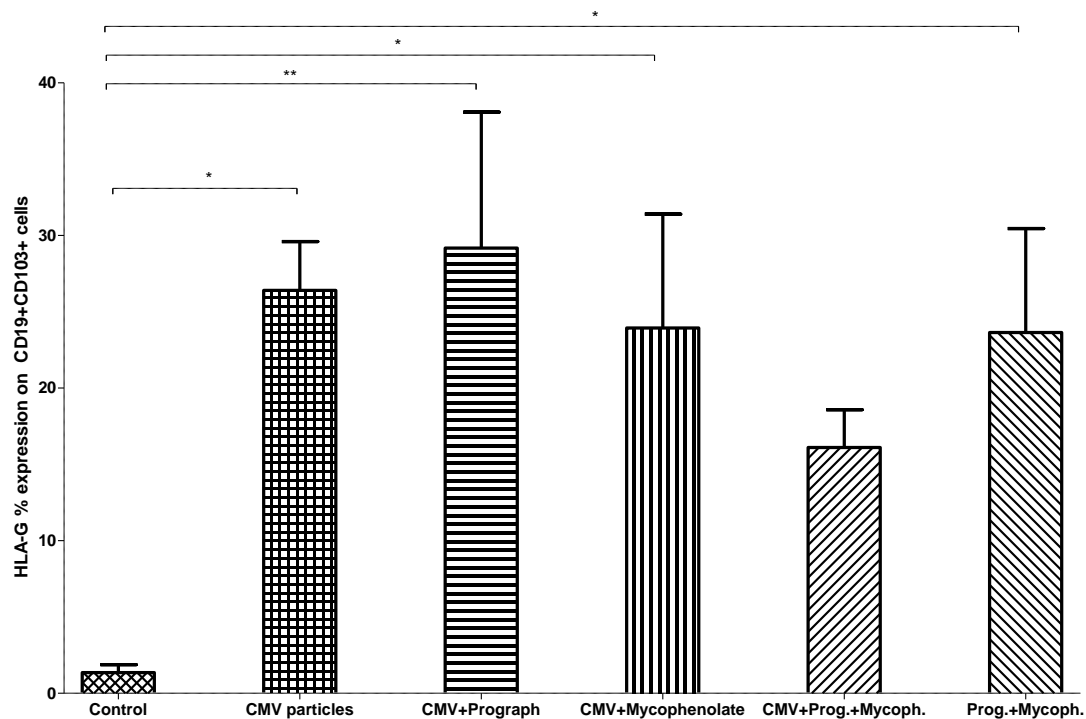


Fig. 8.16. The expression of HLA-G on CD19+CD103+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, * $P<0.05$, ** $P<0.01$.

8.3.4. HLA-G expression by CD3+CD56+CD103+ cells following immunosuppressive drug treatment

In the same way, following on from the previous graph HLA-G was assessed on CD3+CD56+CD103 cells after culture with immunosuppressive drugs for a week (Fig. 8.17). The proportion of HLA-G on those cells following treatment was highest on Prograf + Mycophenolate 35.07% vs 1.94% in control, while the lowest expresser among the treatments was CMV particles + Mycophenolate 5.49%. The other treatments had the following descending proportions: CMV particles 13.83%, CMV particles + Prograf 13.72% and CMV + Prograf + Mycophenolate 7.52%. A comparison of the HLA-G proportions was made among all the treatments and the control using a one-way ANOVA (Dunnett's Multiple Comparison Test) and a statistically significant difference was obtained with overall $P=0.0451$. A significant

value was obtained in treatment with Prograf + Mycophenolate vs the control ($P < 0.05$).

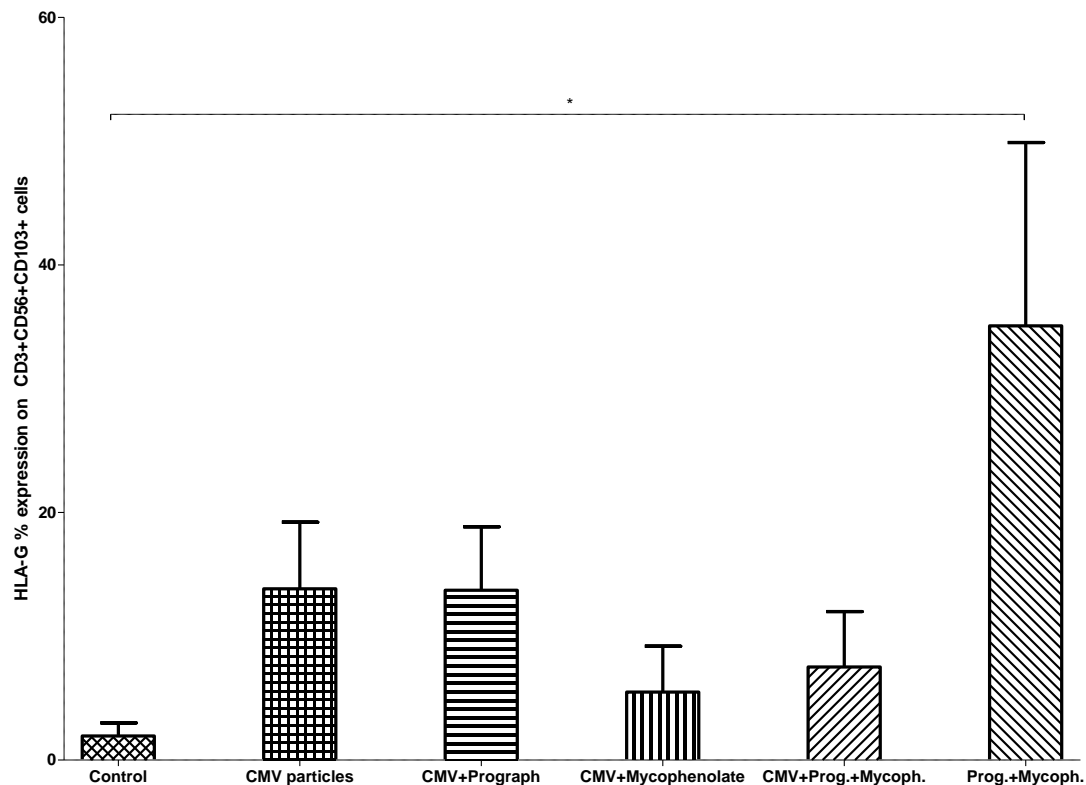


Fig. 8.17. The expression of HLA-G on CD3+CD56+CD103+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $*P < 0.05$.

8.3.5. HLA-G expression by CD56+CD103+ cells following immunosuppressive drug treatment

Finally, CD56+CD103+ expressing cells were studied to assess the proportion of HLA-G expression after cell induction with immunosuppressive drugs for a week (Fig. 8.18). Apparently, all the treatment combinations could induce the expression of HLA-G but to different extents with highest proportion being noted in Prograf + Mycophenolate (21.02%) vs 0.71% in the control. The remainder of the treatments had variable effects on HLA-G expression with the following descending percentages: CMV particles + Prograf 15.23%, CMV particles 10.77%, CMV + Prograf + Mycophenolate 9.80% and CMV particles + Mycophenolate 5.93%. No overall statistical significance was noted after applying a one-way ANOVA (Dunnett's

Multiple Comparison Test; $P=0.1554$), however, a significant difference was recorded in Prograf + Mycophenolate treatment vs control ($P<0.05$).

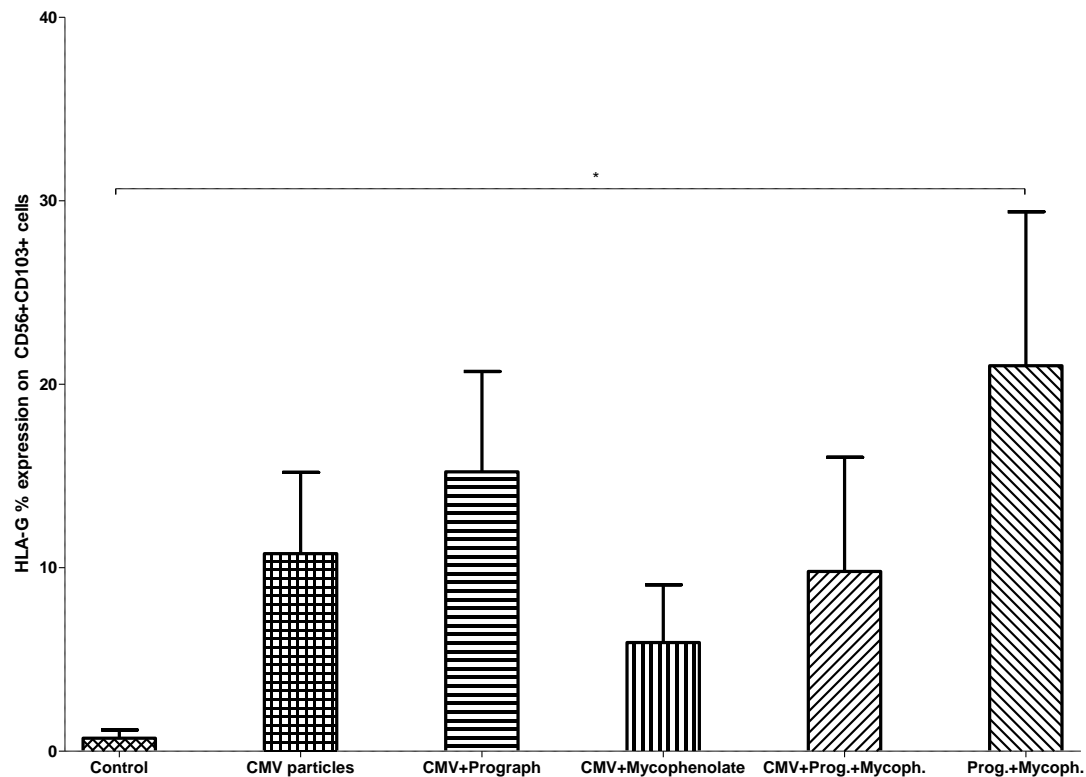


Fig. 8.18. The expression of HLA-G on CD56+CD103+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $*P<0.05$.

8.4. Immunosuppressive drug effects on BAFF expression in cultured PBMCs

In parallel with HLA-G and CD103 assessments following immunosuppressive drug treatment, BAFF antigen expression was also evaluated on the surface of mononuclear cells after treating the PBMCs with physiological concentrations of Prograf and Mycophenolate for seven days. A combination of CMV particles plus Prograf and/or Mycophenolate were used to stimulate the cells in order to evaluate expression of the BAFF molecule on the cultured PBMCs. Healthy subjects ($n = 5$) were involved in this experiment and PBMCs were incubated with a combination of the CMV, Prograf and Mycophenolate in addition to control non-stimulated cells. The

PBMCs following culture were recovered and labelled with mAbs that recognise BAFF antigen and other cell markers including the HLA-G protein.

8.4.1. Immunosuppressive effects on BAFF expression by CD3+CD4+ cells

The BAFF protein was studied on CD3+CD4+ lymphocytes after treating the cells with various combinations of immunosuppressive drugs (Fig. 8.19). Overall, the proportion of BAFF was highest in CMV particles + Mycophenolate 2.12% vs 0.084 in the control, whereas, it was the lowest in Prograf + Mycophenolate 0.23% treatment. The remaining cell populations expressed the following proportions: CMV + Prograf + Mycophenolate 1.11%, CMV particles + Prograf 1.01% and CMV particles 0.86%. After comparing the various treatments with the control, no statistically significant difference was obtained utilizing a one-way ANOVA (Dunnett's Multiple Comparison Test; $P=0.4551$).

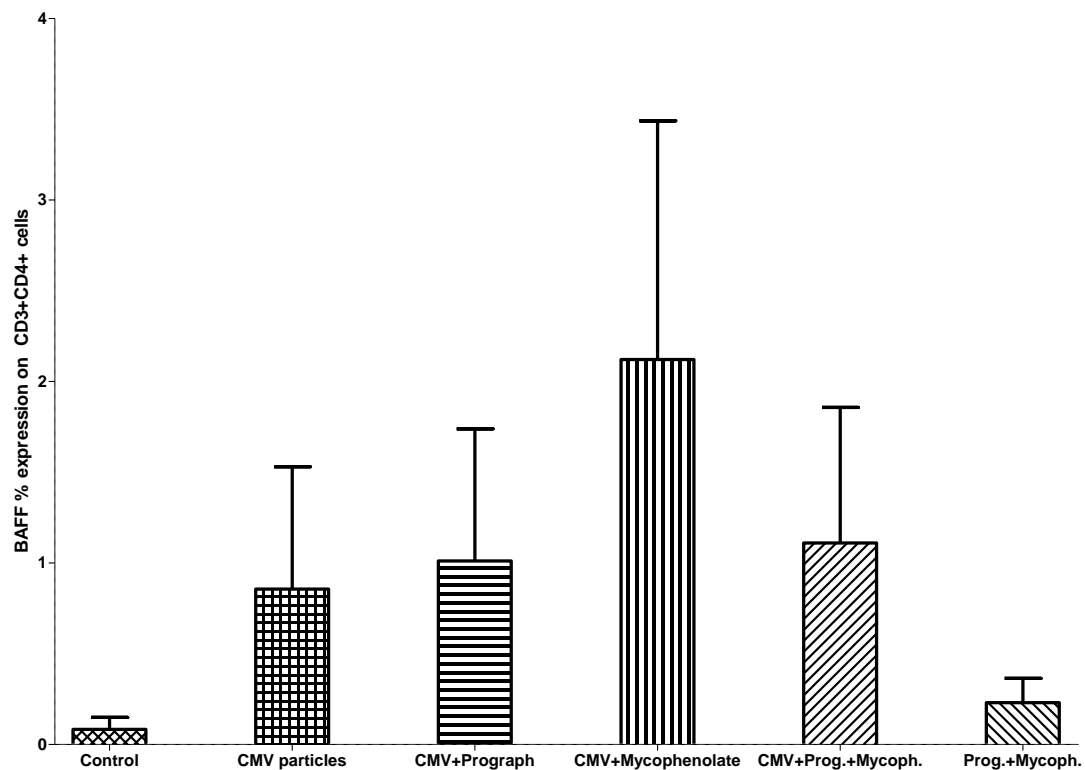


Fig. 8.19. The expression of BAFF on CD3+CD4+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P>0.05$.

8.4.2. Immunosuppressive effects on BAFF expression by CD3+CD8+ lymphocytes

The effect of immunosuppressive drugs on the expression of BAFF protein was assessed on CD3+CD8+ cells after incubation for 7 days (Fig. 8.20). In general, this molecule was slightly upregulated on those cells in all the treatments with the highest proportion recorded in CMV particles + Prograf 9.01% vs 0.25% in the control. The remaining groups expressed BAFF antigen in the following order: Prograf + Mycophenolate 4.44%, CMV particles + Mycophenolate 3.44%, CMV + Prograf + Mycophenolate 2.01% and CMV particles 1.84%. The different treatments were compared with the control utilizing a one-way ANOVA (Dunnett's Multiple Comparison Test) with no statistically significant differences yielded ($P=0.5131$).

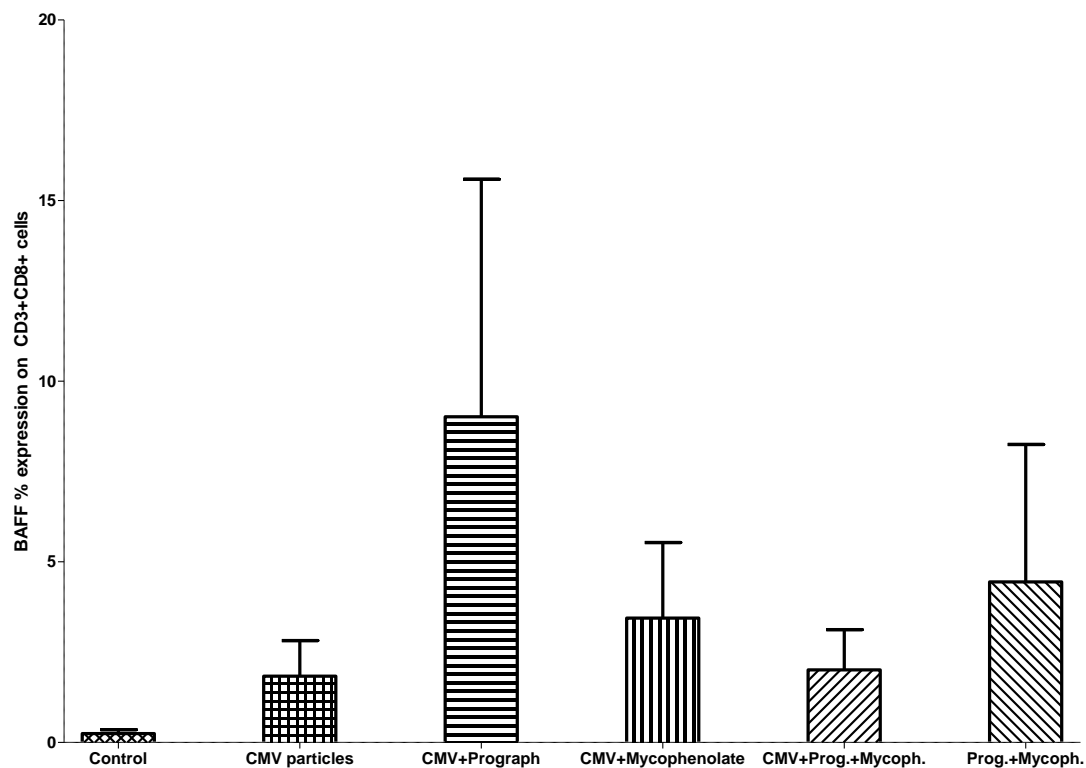


Fig. 8.20. The expression of BAFF on CD3+CD8+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.4.3. Immunosuppressive effects on BAFF expression by CD19+ cells following culture

Similar to above pattern, the expression of the BAFF molecule was measured on CD19+ cells after immunosuppressive medication treatments for a week in whole PBMC culture (Fig. 8.21). There were patterns of upregulation of the BAFF protein with the highest proportion of BAFF antigen expressed in CMV particles + Mycophenolate 7.32%, CMV particles 6.98% and CMV particles + Prograf 6.96%, while the other treatments had slightly increased proportions of BAFF+ cells (2.76% in Prograf + Mycophenolate and 2.60% in CMV + Prograf + Mycophenolate) than in the control 1.67%. A comparison between the treatments and the control was made through a one-way ANOVA (Dunnett's Multiple Comparison Test) and no statistically significant difference was noted ($P=0.2689$).

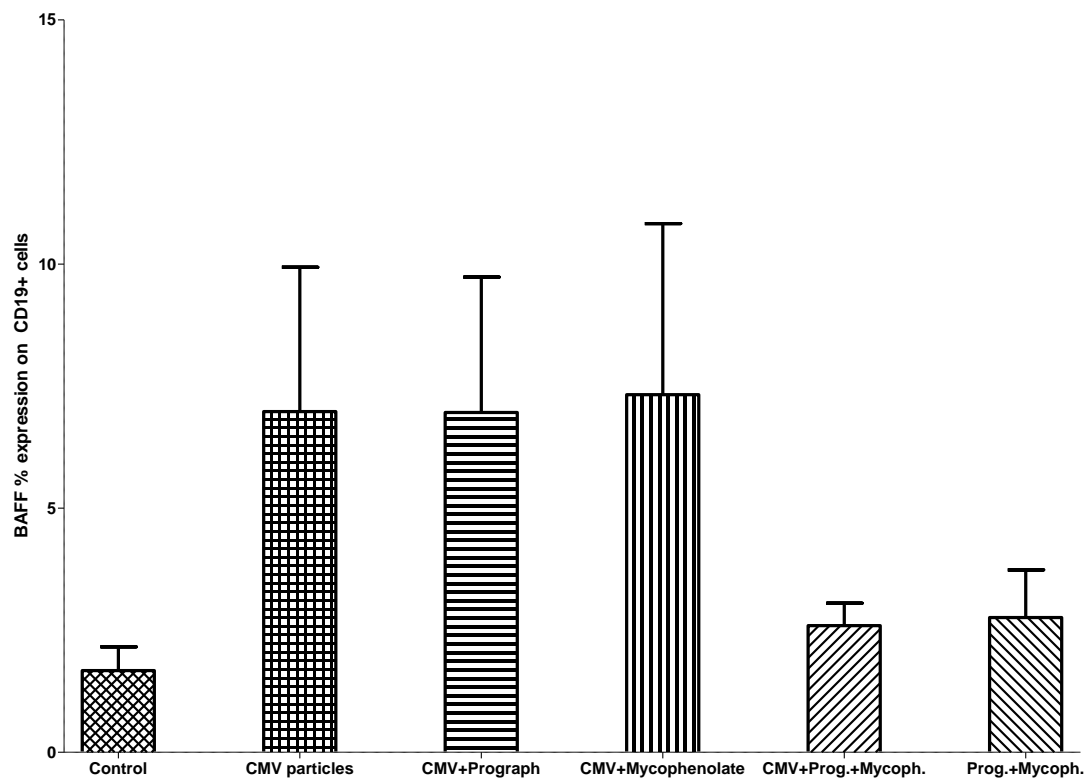


Fig. 8.21. The expression of BAFF on CD19+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P>0.05$.

8.4.4. Immunosuppressive effects on BAFF expression by CD14+HLA-DR+ cells

Likewise, the BAFF protein was measured on CD14+HLA-DR+ cells after culture with the immunosuppressive drug combinations (Fig. 8.22). Most of the treatments had no effect on upregulating the BAFF protein on CD14+HLA-DR+ cells apart from CMV particles which showed a slight increase at 12.33% compared to the control 9.33%; the remaining treatments had lower proportions than the control (Prograf + Mycophenolate 4.85%, CMV particles + Prograf 1.64%, CMV particles + Mycophenolate 1.61% and CMV + Prograf + Mycophenolate 1.36%). No statistically significant difference was noted when the comparison was made with a one-way ANOVA (Dunnett's Multiple Comparison Test; $P=0.2650$).

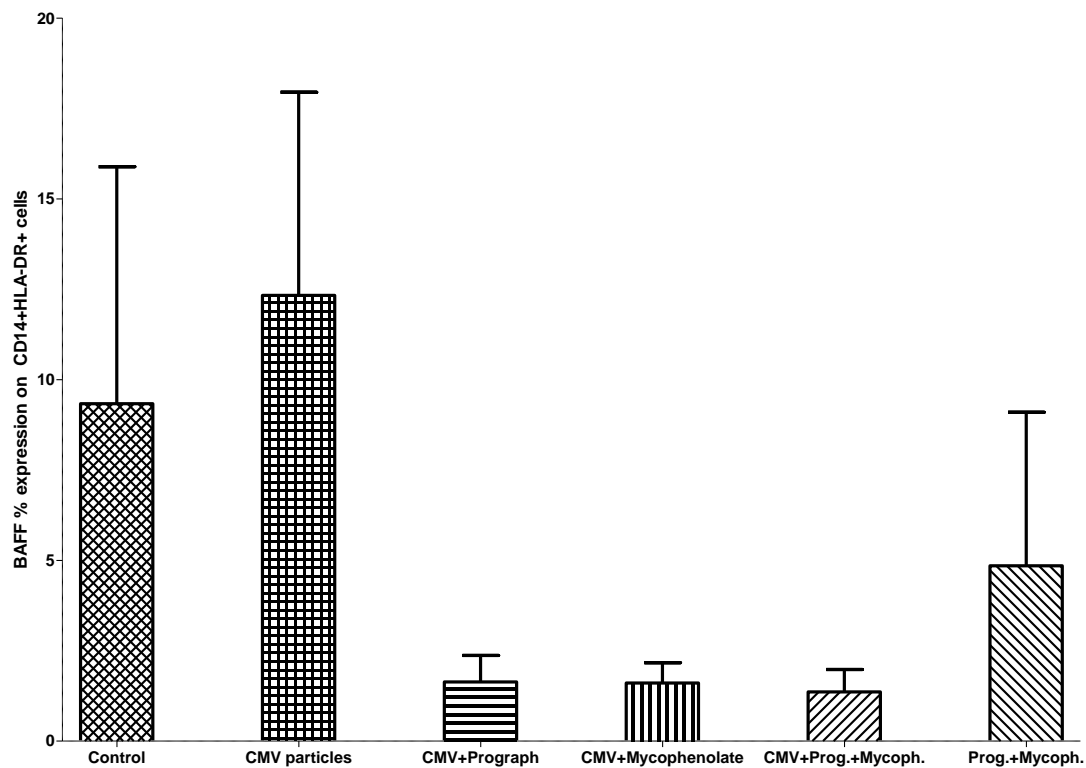


Fig. 8.22. The expression of BAFF on CD14+HLA-DR+ cells following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P>0.05$.

8.4.5. Immunosuppressive effects on BAFF expression by CD3+CD56+ cells following culture

Unlike CD14+HLA-DR+ cells, CD3+CD56+ lymphocytes demonstrated fairly high proportions of BAFF molecule expression after inducing the cells with different combination of immunosuppressive drugs (Fig. 8.23). Overall, the proportion of BAFF+ cells was highest in CMV particles + Mycophenolate 24.78% compared to 7.94% in control, whereas the lowest proportions of BAFF expression was observed in CMV + Prograf + Mycophenolate 9.24% and Prograf + Mycophenolate 9.56% treatments. The other treatments (CMV particles and CMV particles + Prograf) have recorded proportions of 19.07% and 16.76%. From a statistical point of view, no significant difference was obtained after applying a one-way ANOVA (Dunnett's Multiple Comparison Test; $P=0.4148$).

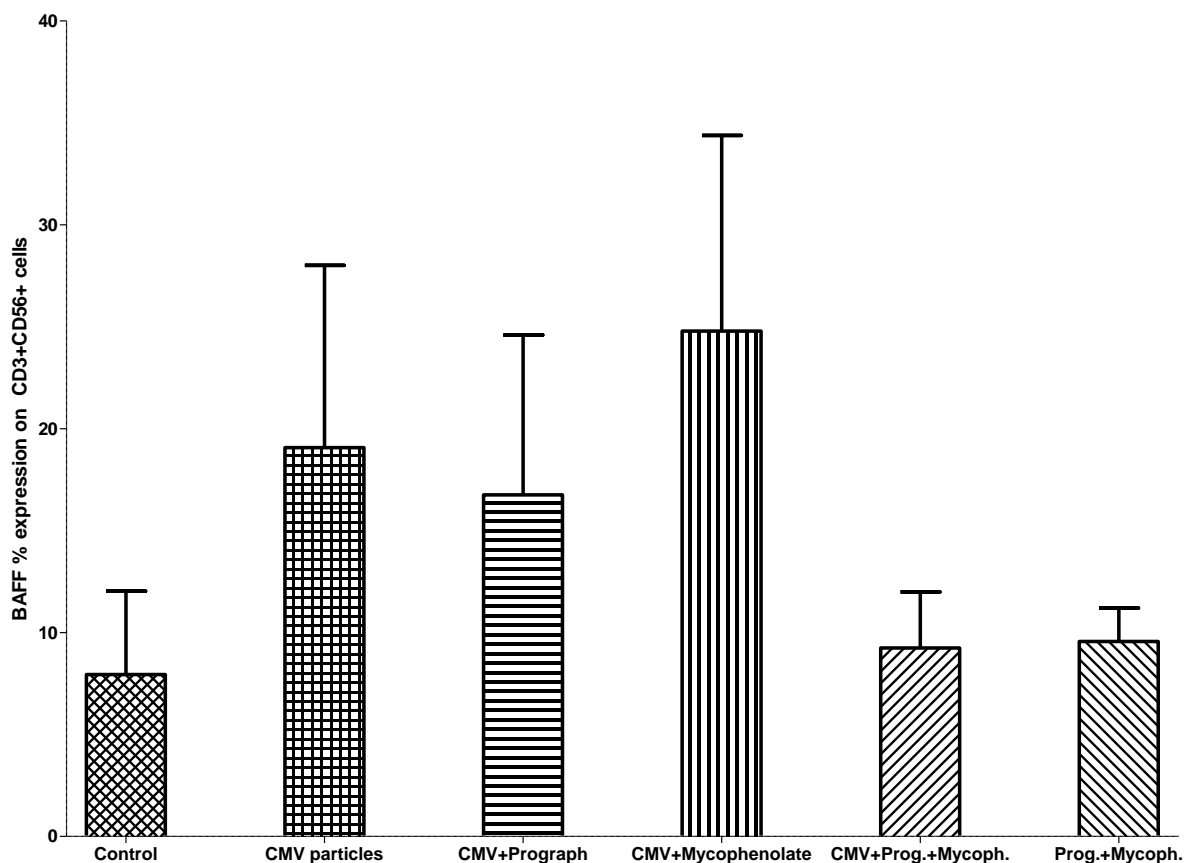


Fig. 8.23. The expression of BAFF on CD3+CD56+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P>0.05$.

8.4.6. Immunosuppressive effects on BAFF expression by CD56+ cells

Finally, CD56+ cells were involved in the assessment of BAFF after treating the cells with different combinations of immunosuppressive drugs (Fig. 8.24). In general, these cells expressed increasing proportions of BAFF+ cells in all the treatment combinations with the highest percentage in descending order: CMV particles + Prograf 10.35%, CMV particles + Mycophenolate 8.45%, CMV particles 8.20%, CMV + Prograf + Mycophenolate 6.36% and Prograf + Mycophenolate 6.26%, while in the control the proportion of CD56+ cells expressing BAFF was 0.76%. The treatment combinations were compared with the control using a one-way ANOVA (Dunnett's Multiple Comparison Test) and no statistically significant difference was obtained ($P = 0.6357$).

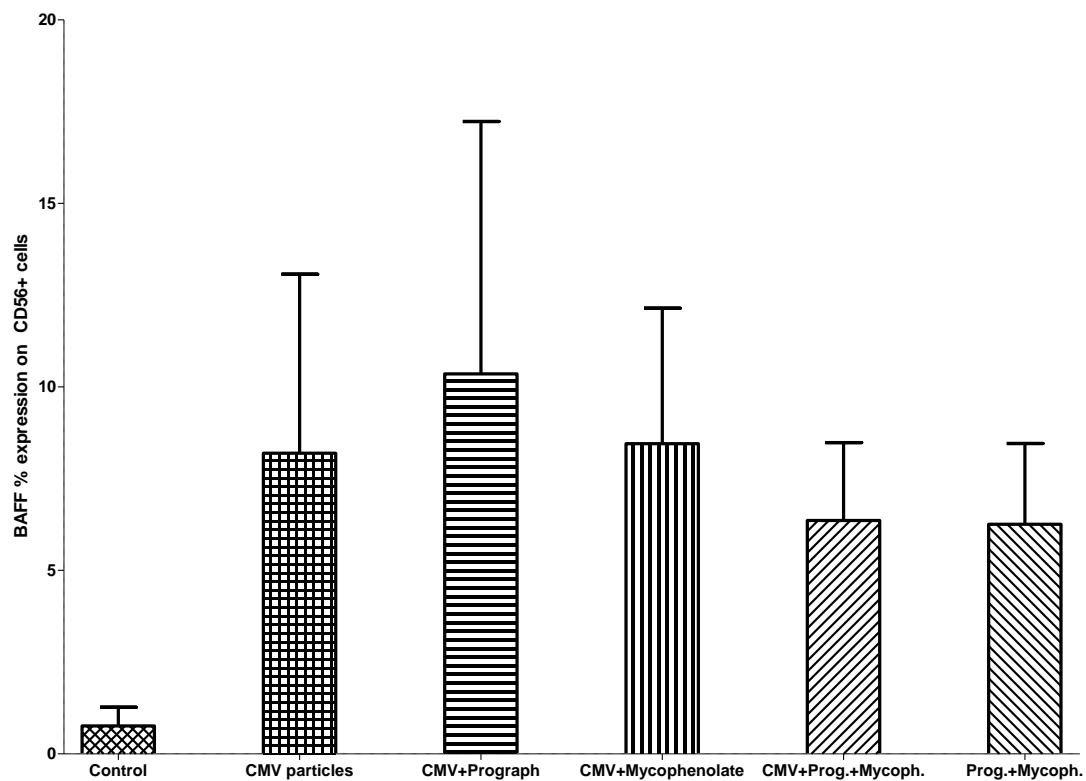


Fig. 8.24. The expression of BAFF on CD56+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.5. Immunosuppressive drug effects on HLA-G expression by BAFF+ PBMCs after culture

The expression of HLA-G on PBMC populations expressing the BAFF antigen was evaluated after treating the mononuclear cells with different combinations of immunosuppressive drugs in order to establish a link between the BAFF positive cells and the co-expression of HLA-G molecule on them. The cells were phenotyped with mAbs specific for HLA-G, BAFF and other mononuclear subpopulations, cells were acquired with an Accuri C6 flow cytometry and data were analysed with the software provided.

8.5.1. Expression of HLA-G by CD3+CD4+BAFF+ cells following in vitro treatment with immunosuppressive drugs

The expression of HLA-G on CD3+CD4+BAFF+ cells was assessed after culture with immunosuppressive medication in various combinations (Fig. 8.25). In general, the proportion of HLA-G+ cells were increased in all the treatments with highest percentage in CMV particles + Prograf 23.18% vs 0.74% in control, while the lowest proportion was recorded in CMV particle treatment (11.67%). The remaining treatments demonstrated the following descending proportions: CMV + Prograf + Mycophenolate 20.66%, CMV particles + Mycophenolate 15.49% and Prograf + Mycophenolate 14.35%. After comparing the treatments with control, no statistically significant difference was obtained ($P=0.2452$) when a one-way ANOVA (Dunnett's Multiple Comparison Test) was applied.

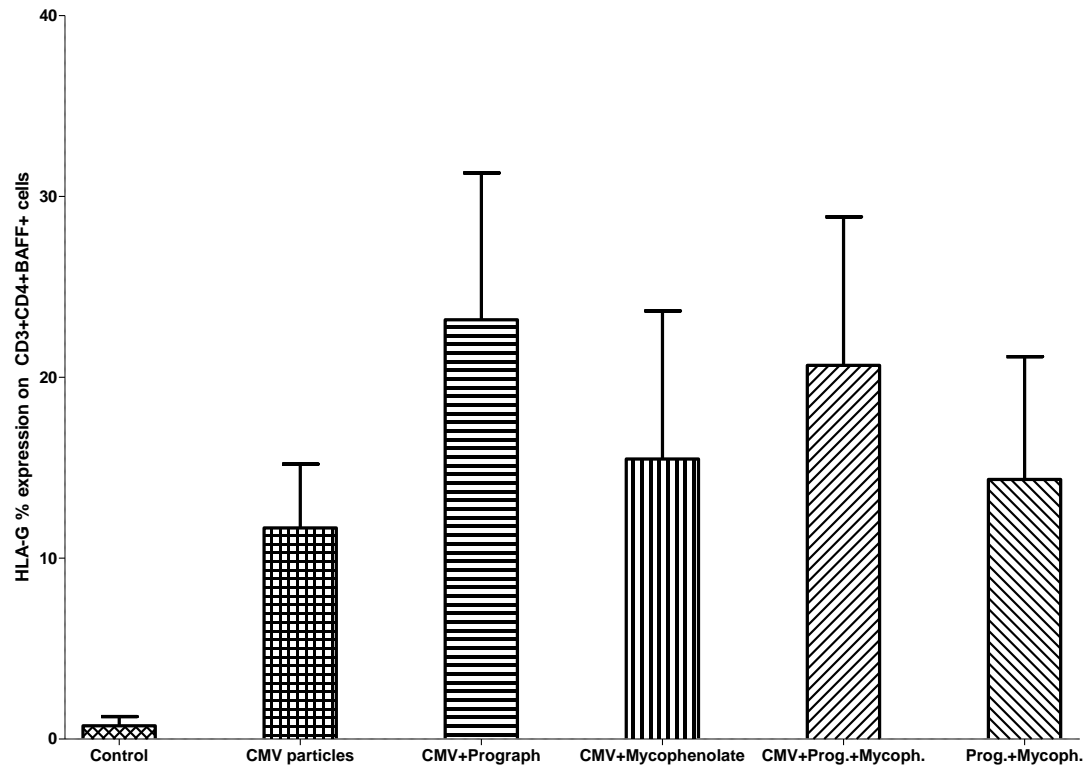


Fig. 8.25. The expression of HLA-G on CD3+CD4+BAFF+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.5.2. Expression of HLA-G by CD3+CD8+BAFF+ cells following immunosuppressive drug treatment

Likewise, HLA-G expression on CD3+CD8+BAFF+ cells was evaluated following immunosuppressive induction for a week (Fig. 8.26) and as demonstrated below, HLA-G was upregulated on all the treatment combinations with highest proportions observed in CMV + Prograf + Mycophenolate 14.95%, CMV particles + Mycophenolate 12.73%, CMV particles 11.38%, Prograf + Mycophenolate 10.59% and CMV particles + Prograf 9.57%, while the HLA-G percentage was 0.90% in control. The groups showed no statistically significant difference when a one-way ANOVA was performed ($P=0.6212$).

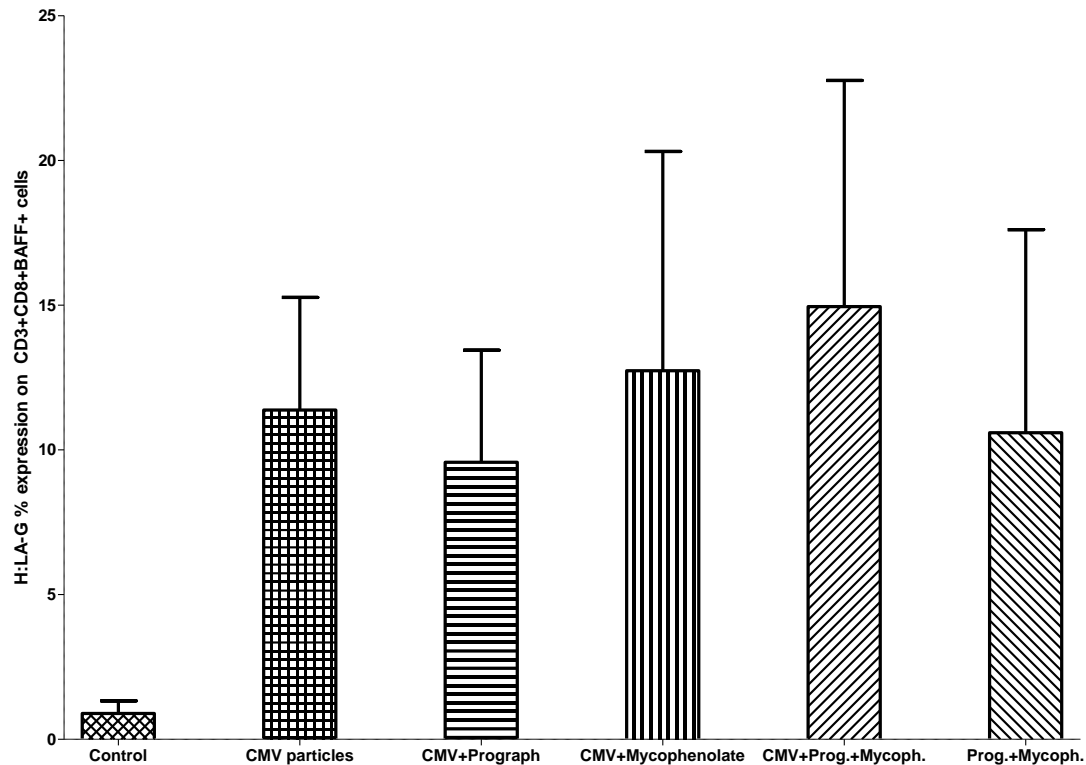


Fig. 8.26. The expression of HLA-G on CD3+CD8+BAFF+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.5.3. HLA-G expression by CD19+BAFF+ cells following immunosuppressive drug treatment

In the same way, HLA-G expression on CD19+BAFF+ cells were measured after cell culture with immunosuppressive drugs (Fig. 8.27). Clearly, all the various treatments have upregulated HLA-G with proportions in the following descending order: CMV + Prograf + Mycophenolate 12.74%, CMV particles + Mycophenolate 11.95%, CMV particles 10.48%, CMV particles + Prograf 8.76% and Prograf + Mycophenolate 7.95%. When the groups were compared with the control using one-way ANOVA (Dunnett's Multiple Comparison Test), no statistically significant difference was obtained ($P=0.5719$).

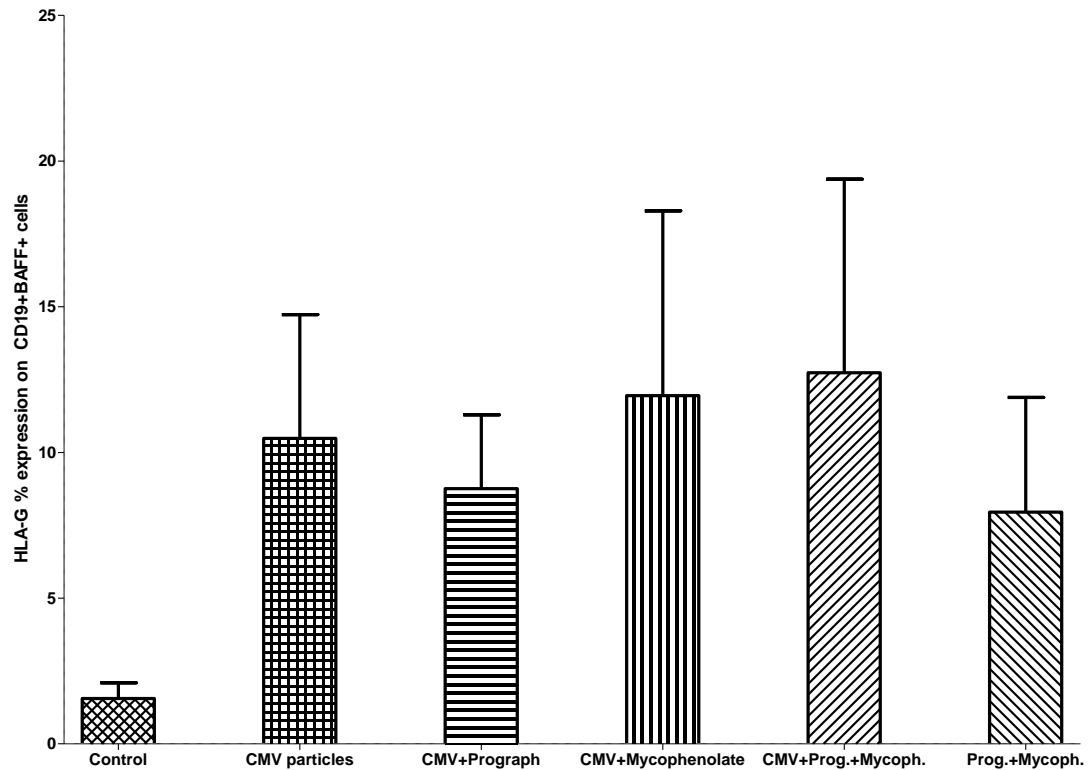


Fig. 8.27. The expression of HLA-G on CD19+BAFF+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.5.4. HLA-G expression by CD14+HLA-DR+BAFF+ cells following immunosuppressive drug treatment

The expression of HLA-G on CD14+HLA-DR+BAFF+ cells was estimated after culture with immunosuppressive drugs (Fig. 8.28). the proportion of HLA-G+ cells was increased in some of the treatments (CMV particles 7.634%, CMV particles + Mycophenolate 3.65% and Prograf +Mycophenolate 4.33%. Unlike, the remaining treatments were the was no upregulation of the HLA-G expression noted (CMV particles + Prograf 0.87%, control 0.67% and CMV + Prograf + Mycophenolate 0.22%). No statistically significant difference was recorded upon performing a one-way ANOVA (Dunnett's Multiple Comparison Test; $P = 0.6545$).

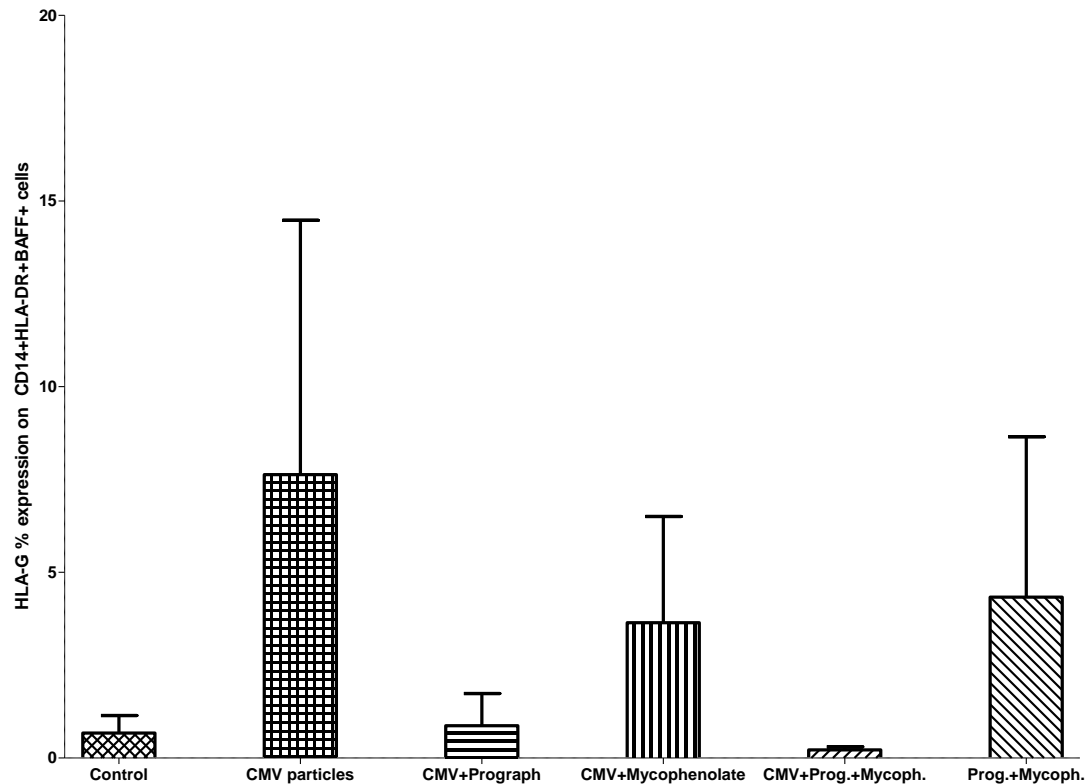


Fig. 8.28. The expression of HLA-G on CD14+HLA-DR+BAFF+ cells following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.5.5. HLA-G expression by CD3+CD56+BAFF+ cells following immunosuppressive drug treatment

Similar to the above, the influence of immunosuppressive drugs was assessed on HLA-G expression on CD3+CD56+BAFF+ cells after culture with various drug combinations (Fig. 8.29). Apparently, all the treatment combinations upregulated HLA-G expression with the highest proportions recorded in the following descending patterns; CMV particles + Prograf 39.26%, CMV + Prograf + Mycophenolate 35.70%, Prograf + Mycophenolate 25.37%, CMV particles 24.44%, CMV particles + Mycophenolate 16.51% and 1.56% in the control. The comparison between the treatments and the control demonstrated close to significant difference with overall $P = 0.0808$ when a one-way ANOVA (Dunnett's Multiple Comparison Test) was applied. In particular, there was a significant difference between CMV particles + Prograf vs control.

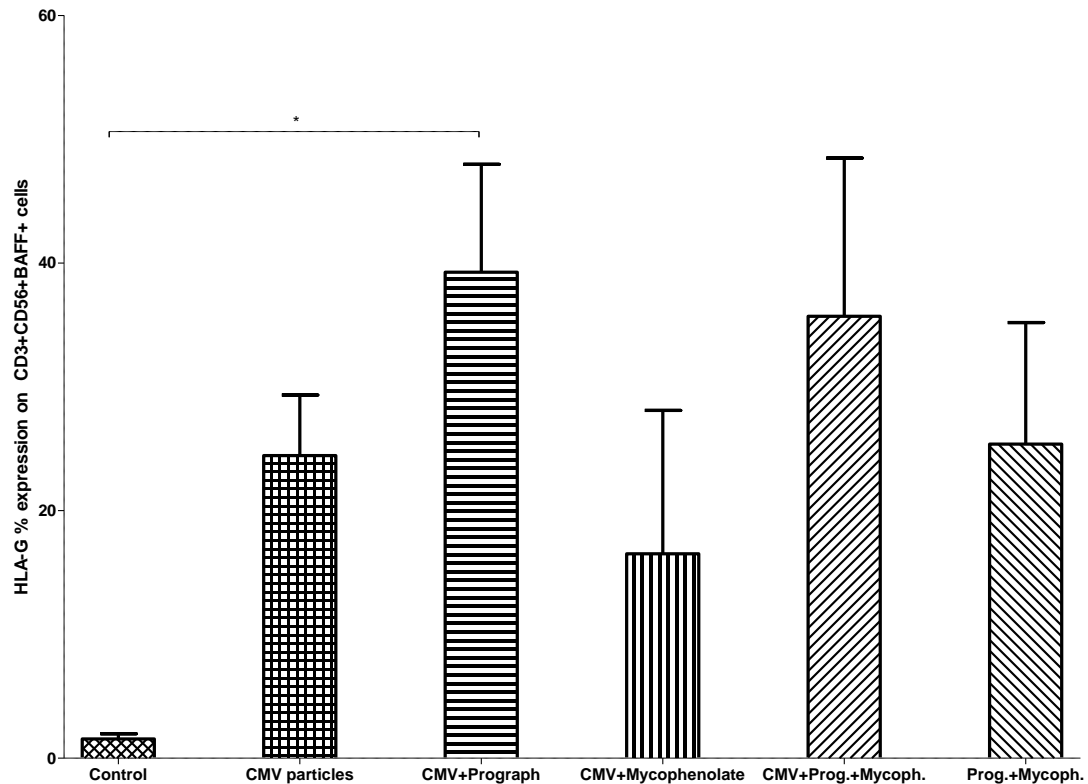


Fig. 8.29. The expression of HLA-G on CD3+CD56+BAFF+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, * $P < 0.05$.

8.5.6. HLA-G expression by CD56+BAFF+ cells following immunosuppressive drug treatment

Finally, in CD56+BAFF+ cells the expression of HLA-G was measured after culture with immunosuppressive drugs for a week (Fig. 8.30) and as depicted, all the groups of treatments had increased expression of HLA-G with proportions ranked in the following order: CMV particles + Prograf 29.93%, CMV particles + Mycophenolate 26.12%, CMV particles 25.58%, CMV + Prograf + Mycophenolate 18.65%, Prograf +Mycophenolate 15.86% and 0.76 % in the control. After comparing the groups with control utilizing one-way ANOVA (Dunnett's Multiple Comparison Test), no statistically significant difference was recorded ($P=0.1693$).

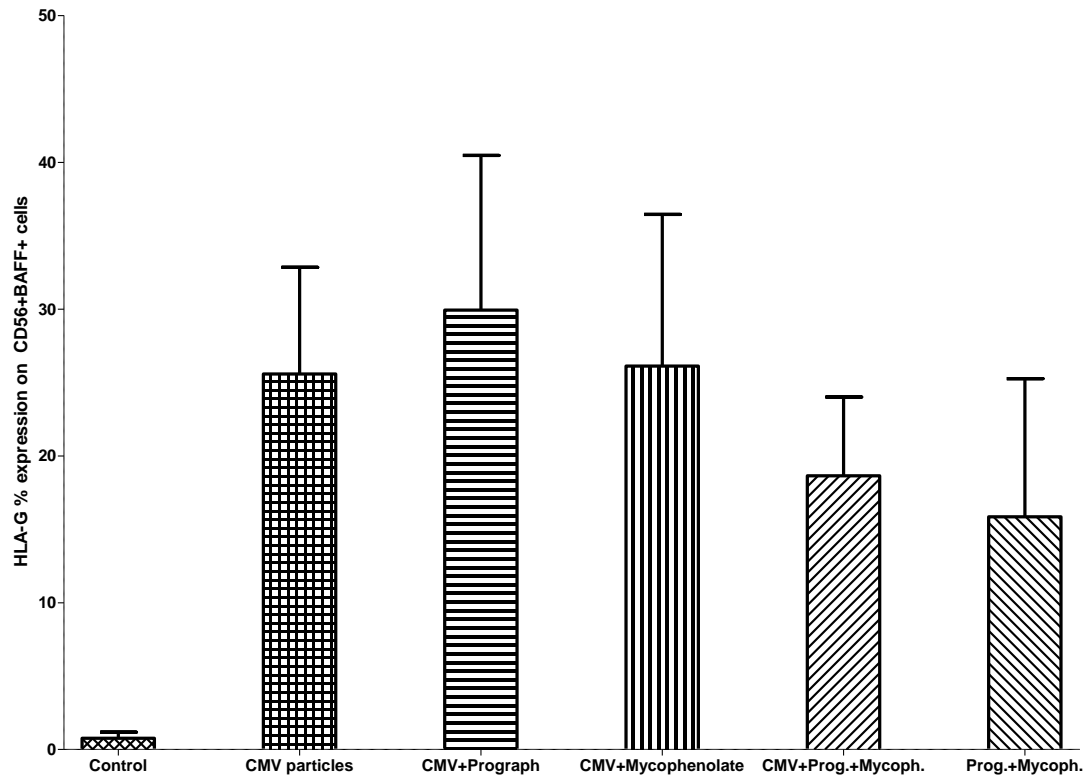


Fig. 8.30. The expression of HLA-G on CD56+BAFF+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.6. Immunosuppressive drug effects on BAFF-R expression in cultured PBMCs

PBMCs extracted from five healthy subjects were treated with immunosuppressive drugs (Prograf and Mycophenolate and CMV particles) in five different combinations. The PBMCs were incubated for seven days, after which the cells were labelled with mAbs to assess the response of BAFF-R protein on several mononuclear subpopulations in parallel with HLA-G and the remaining basic CD markers specific for each subpopulation. Data obtained from flow cytometry were analysed utilising Accuri C6 software.

8.6.1. Immunosuppressive effects on BAFF-R expression by CD3+CD4+ cells

The effect of immunosuppressive drugs induction on CD3+CD4+ cells was studied to assess the expression of BAFF-R protein on these cells (Fig. 8.31). Overall, this protein was upregulated in response to various combinations of immunosuppressive treatments with proportions ranging as follows: CMV particles + Prograf 16.58%, CMV particles 16.41%, CMV particles + Mycophenolate 15.38%, Prograf + Mycophenolate 15.13%, CMV + Prograf + Mycophenolate 10.36% and 3.260% in the control. No statistically difference was noted after a one-way ANOVA (Dunnett's Multiple Comparison Test) was performed ($P=0.1105$).

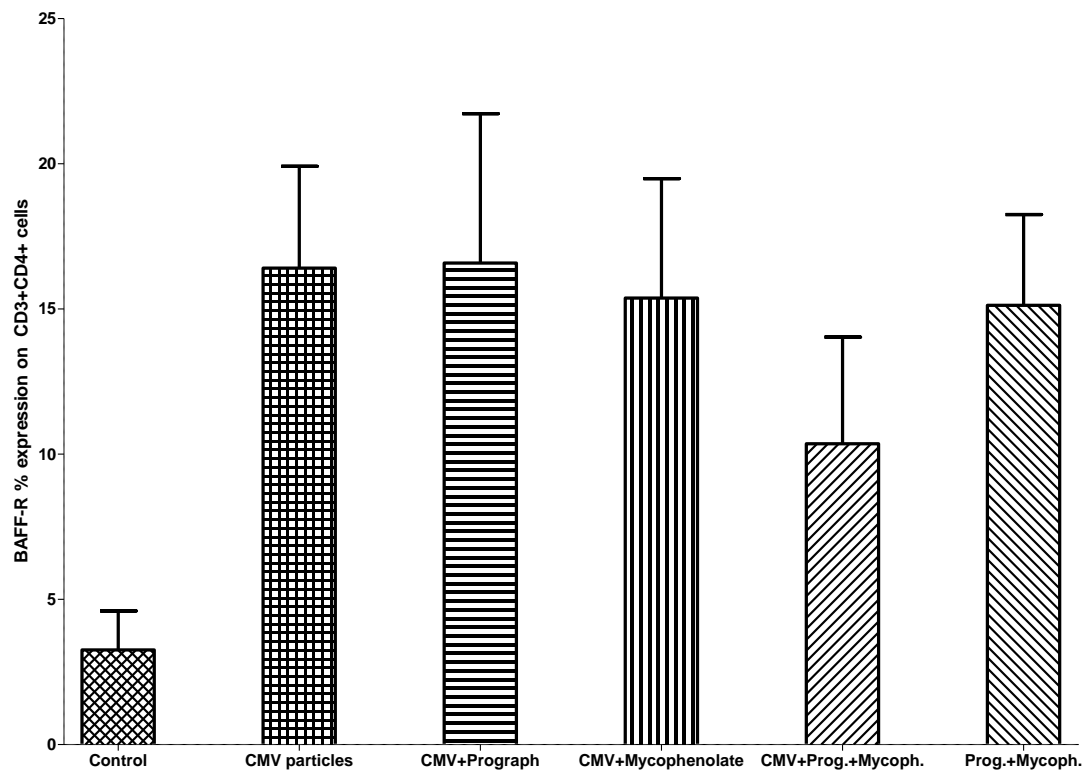


Fig. 8.31. The expression of BAFF-R on CD3+CD4+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.6.2. Immunosuppressive effects on BAFF-R expression by CD3+CD8+ cells

Likewise, the BAFF-R protein was evaluated on CD3+CD8+ cells after immunosuppressive drugs been incubated in whole PBMC culture for a week (Fig. 8.32). As shown below, almost all the treatments had little or no effects on the proportion of BAFF-R expression (Prograf + Mycophenolate 31.51%, CMV particles + Prograf 31.38%, control 29.10% CMV + Prograf + Mycophenolate 29.09%, CMV particles + Mycophenolate 25.42%) with the exception of CMV particles treatment which showed the highest effect on the proportion of BAFF-R+ cells (41.04%). After the groups were compared with one-way ANOVA (Dunnett's Multiple Comparison Test), no statistically significant difference was recorded ($P=0.7875$).

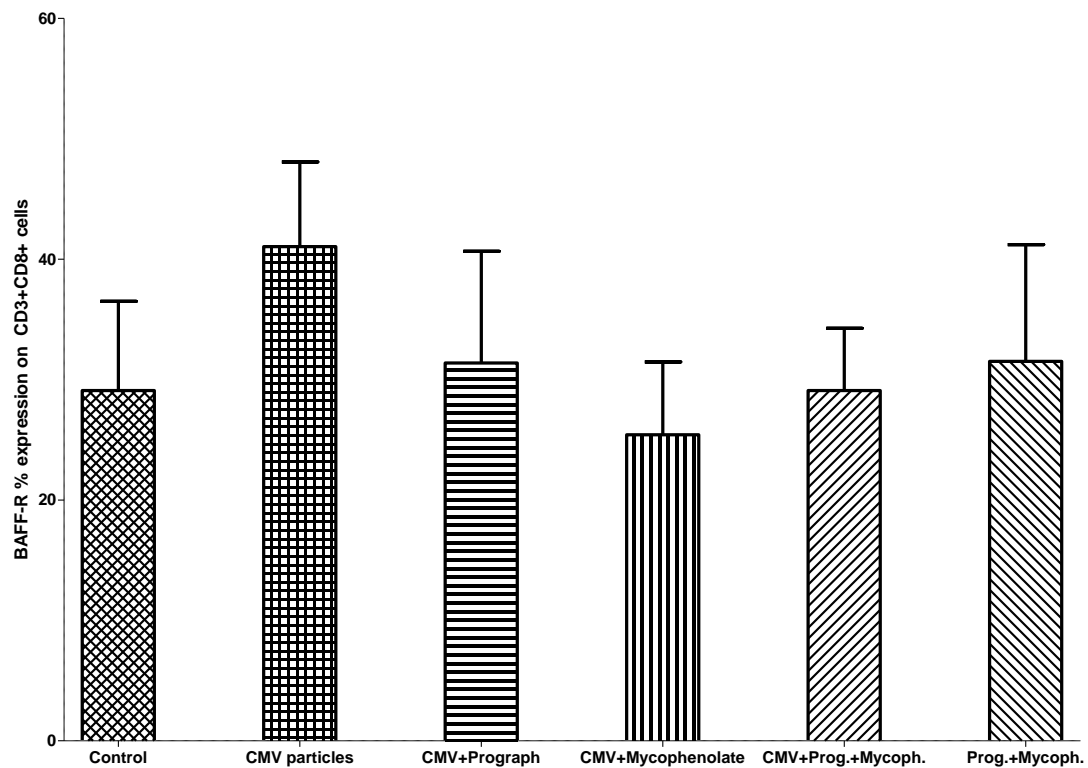


Fig. 8.32. The expression of BAFF-R on CD3+CD8+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.6.3. Immunosuppressive effects on BAFF-R expression by CD19+ cells

The expression of BAFF-R was also evaluated on CD19+ cells after treating PBMC with immunosuppressive medications for a week (Fig. 8.33). Almost all the groups of treatments increased the proportion of BAFF-R in response to immunosuppressive drugs with relatively close proportions (CMV particles + Prograf 91.18%, Prograf + Mycophenolate 88.85%, CMV particles + Mycophenolate 88.79%, CMV particles 81.29%, CMV + Prograf + Mycophenolate 78.00% and control 72.57%). There was no statistically significant difference when a one-way ANOVA was applied (Dunnett's Multiple Comparison Test; $P=0.8370$).

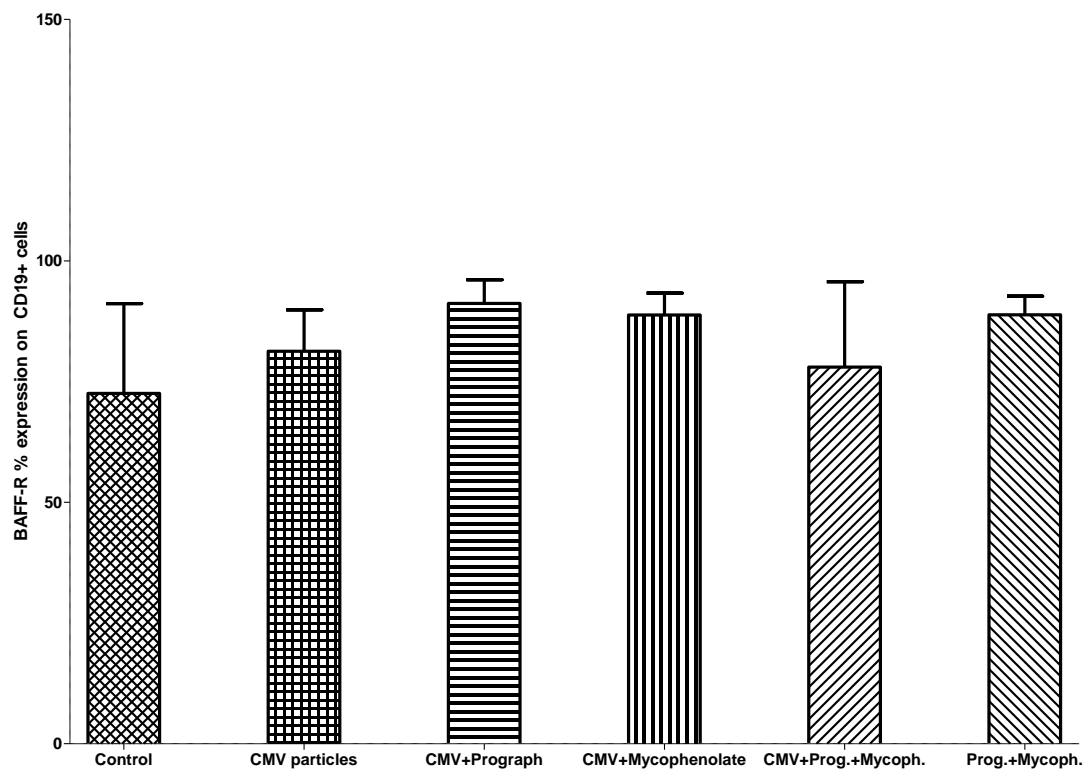


Fig. 8.33. The expression of BAFF-R on CD19+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P>0.05$.

8.6.4. Immunosuppressive effects on BAFF-R expression by CD14+HLA-DR+ cells

The BAFF-R molecule was tested on CD14+HLA-DR+ cells after incubation with immunosuppressive drugs for a week (Fig. 8.34). As it is depicted below, none of the treatments increased BAFF-R expression on CD14+HLA-DR+ cells, instead all of them have down regulated the molecule after culture. The following descending proportions of BAFF-R+ cells were recorded in each group (control 22.29%, CMV particles + Prograf 11.19% CMV + Prograf + Mycophenolate 9.20%, CMV particles 8.30%, CMV particles + Mycophenolate 7.47%, Prograf +Mycophenolate 5.18%). Upon comparing the groups with a one-way ANOVA (Dunnett's Multiple Comparison Test), no statistically significant difference was obtained ($P=0.7829$)

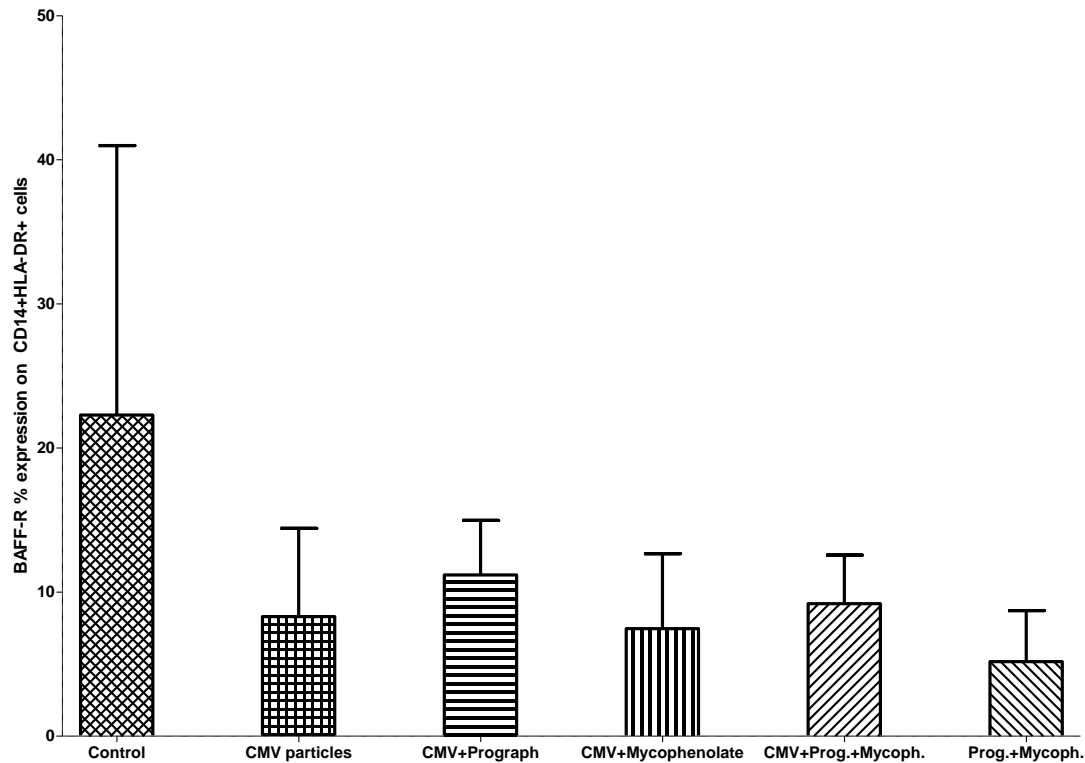


Fig. 8.34. The expression of BAFF-R on CD14+HLA-DR+ cells following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P>0.05$.

8.6.5. Immunosuppressive effects on BAFF-R expression by CD3+CD56+ cells

Conversely, BAFF-R expression on CD3+CD56+ following culture with immunosuppressive medications (Fig. 8.35) revealed upregulation of the protein on all the treated combinations with highest proportions in CMV particles + Mycophenolate 21.95%, CMV particles 20.53% and CMV particles + Prograf 18.53%. This was followed by the remaining treatments (CMV + Prograf + Mycophenolate 13.83%, Prograf + Mycophenolate 10.20% and control 4.11%). There was no statistically significant difference between the groups but the overall P value was close to significance ($P=0.0834$) when a one-way ANOVA was applied (Dunnett's Multiple Comparison Test), particularly CMV particles + Mycophenolate treatment vs control had a significant difference ($*P<0.05$).

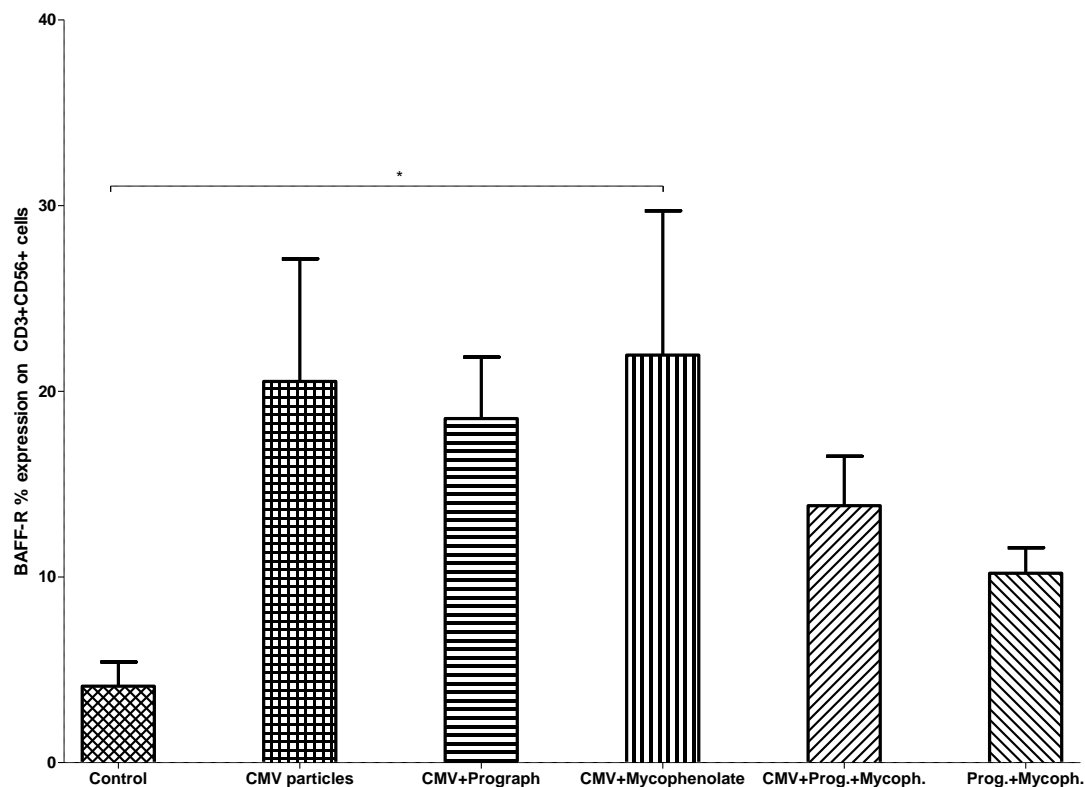


Fig. 8.35. The expression of BAFF-R on CD3+CD56+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $*P<0.05$.

8.6.6. Immunosuppressive effects on BAFF-R expression by CD56+ cells

Lastly, the expression of BAFF-R protein was also assessed on CD56+ cells after culture with immunosuppressive medications for 7 days (Fig. 8.36) and all the treated combinations induced the expression of BAFF-R on CD56+ cells. The proportions cells expressing the BAFF-R molecule were arranged in the following descending manner: CMV particles 32.57%, CMV particles + Mycophenolate 29.28%, CMV + Prograf + Mycophenolate 26.36%, CMV particles + Prograf 24.52%, Prograf +Mycophenolate 19.87% and control 17.85%. Upon comparing the groups with the control using a one-way ANOVA (Dunnett's Multiple Comparison Test), no significant difference was noted ($P=0.9174$).

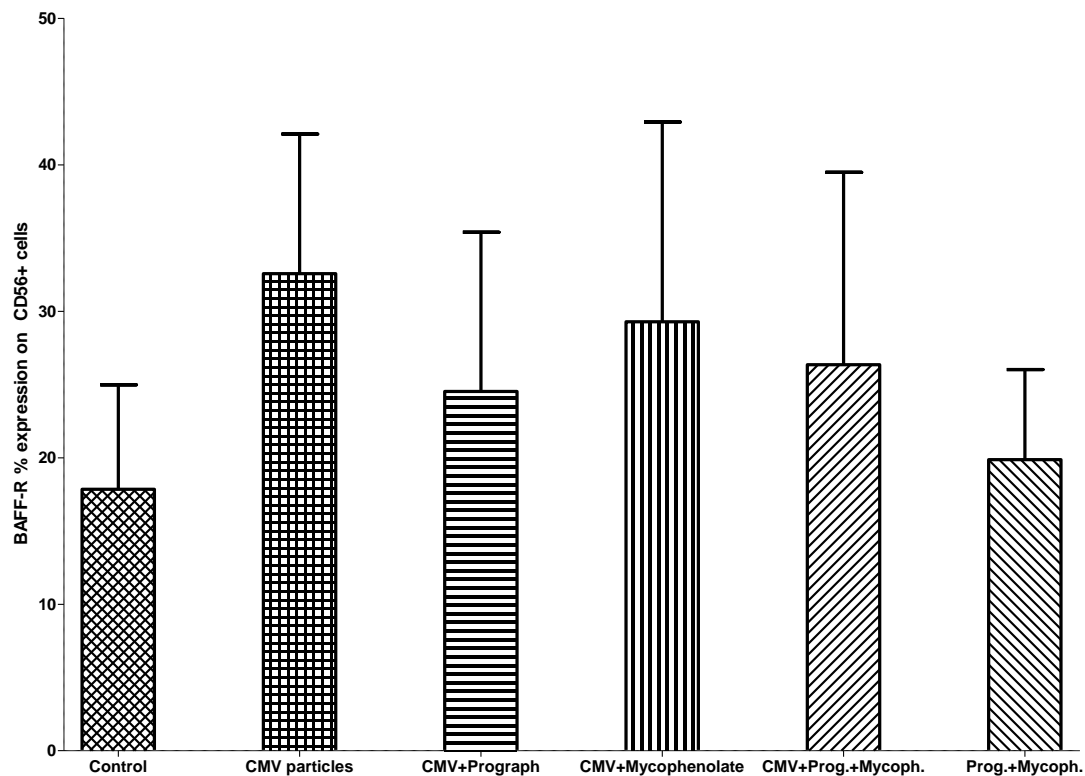


Fig. 8.36. The expression of BAFF-R on CD56+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P>0.05$.

8.7. Immunosuppressive effects on HLA-G expression by BAFF-R+ PBMCs

In parallel with BAFF-R phenotyping, HLA-G was also assessed on PBMC populations that expressed the BAFF-R antigen. Cells were labelled with mAbs and acquired with an Accuri C6 flow cytometer and data were analysed with the software provided.

8.7.1. HLA-G expression by CD3+CD4+BAFF-R+ cells following immunosuppressive drug treatment

HLA-G expression was tested on CD3+CD4+BAFF-R+ cells after stimulation with immunosuppressive medications for a week (Fig. 8.37). All the cells treated with different combinations of immunosuppressive drugs showed increased proportions of BAFF-R+ cells, particularly with CMV particles + Prograf 10.02%, CMV + Prograf + Mycophenolate 9.97% and CMV particles + Mycophenolate 8.52%, while the remaining treatments showed mild increase in the proportion of HLA-G+ cells: CMV particles 3.58%, Prograf + Mycophenolate 2.37% and 1.2% in the control. The different treatments were compared with the control utilizing a one-way ANOVA (Dunnett's Multiple Comparison Test) and there was a significant difference among them ($P=0.0420$).

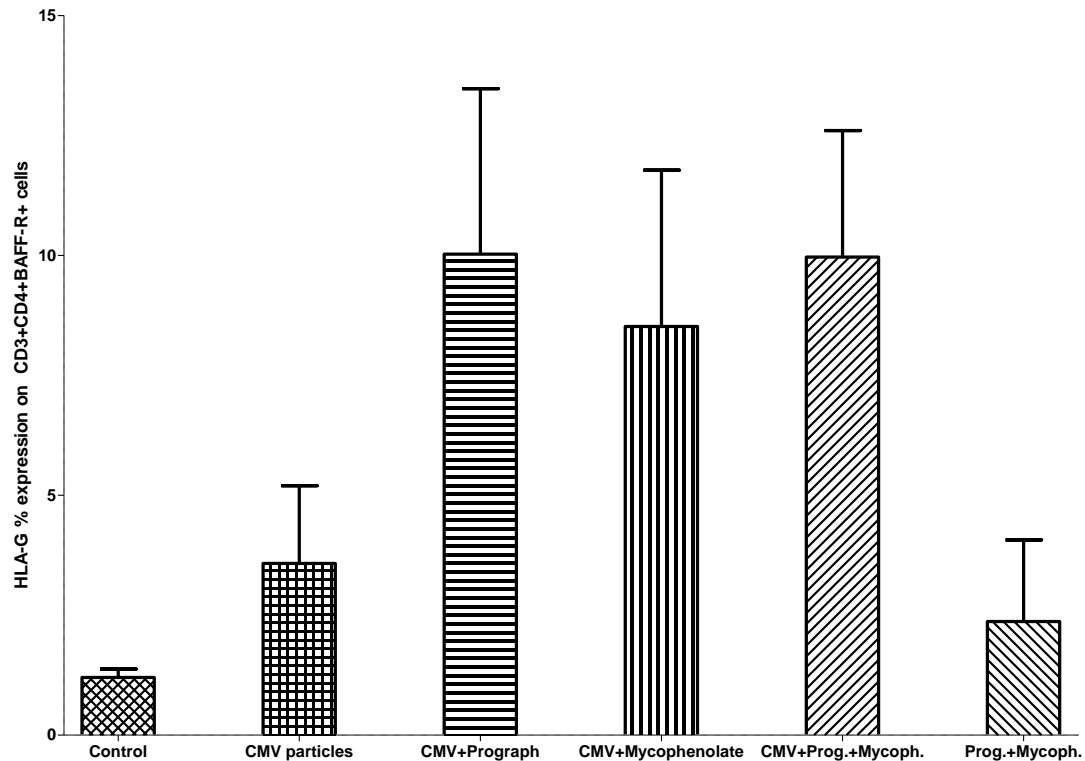


Fig. 8.37. The expression of HLA-G on CD3+CD4+BAFF-R+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, overall $P < 0.05$.

8.7.2. HLA-G expression by CD3+CD8+BAFF-R+ cells following immunosuppressive drug treatment

Likewise, HLA-G co-expression on CD3+CD8+BAFF-R+ cells was measured after treating the cells with immunosuppressive drugs for 7 days (Fig. 8.38) and as illustrated below, treatment with CMV particles + Prograf yielded the highest proportion 6.28%, while the lowest proportions, 0.45% and 0.32% of HLA-G+ cells, were expressed on treatment with Prograf + Mycophenolate and the control, respectively. The other treatments had slightly upregulated expression of HLA-G (CMV particles + Mycophenolate 1.92%, CMV particles 1.49% and CMV + Prograf + Mycophenolate 1.35%). Upon performing a one-way ANOVA (Dunnett's Multiple Comparison Test) the overall P value was close to significance ($P = 0.0775$), in particular, there was a significant difference between CMV particles + Prograf vs control ($*P < 0.05$).

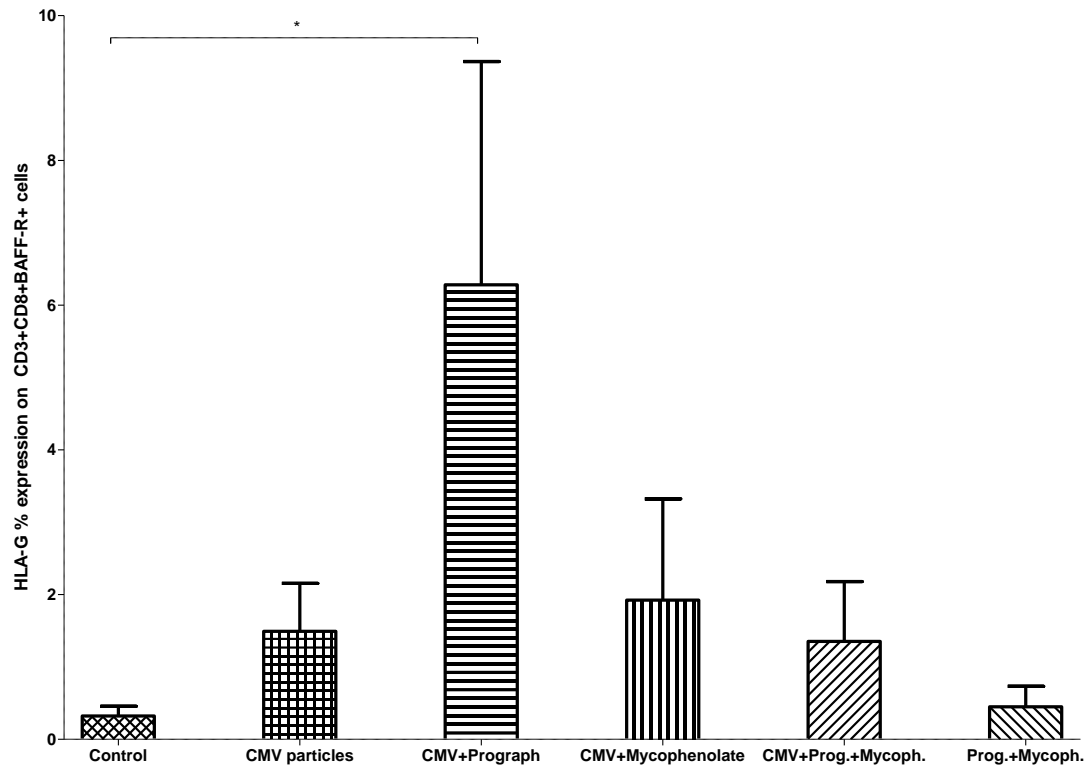


Fig. 8.38. The expression of HLA-G on CD3+CD8+BAFF-R+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, * $P < 0.05$.

8.7.3. HLA-G expression by CD19+BAFF-R+ cells following immunosuppressive drug treatment

On CD19+BAFF-R+ cells, the expression of HLA-G was assessed after culture with immunosuppressive drugs for 7 days (Fig. 8.39) and there was a slight increase in the proportion of HLA-G expressing cells (CMV particles 3.59%, CMV particles + Mycophenolate 2.79%, CMV particles + Prograf 2.22, Prograf + Mycophenolate 0.98%, CMV + Prograf + Mycophenolate 0.71% and 0.74% in the control. When the groups were compared with control with a one-way ANOVA (Dunnett's Multiple Comparison Test) there was no statistically significant difference obtained ($P = 0.2724$).

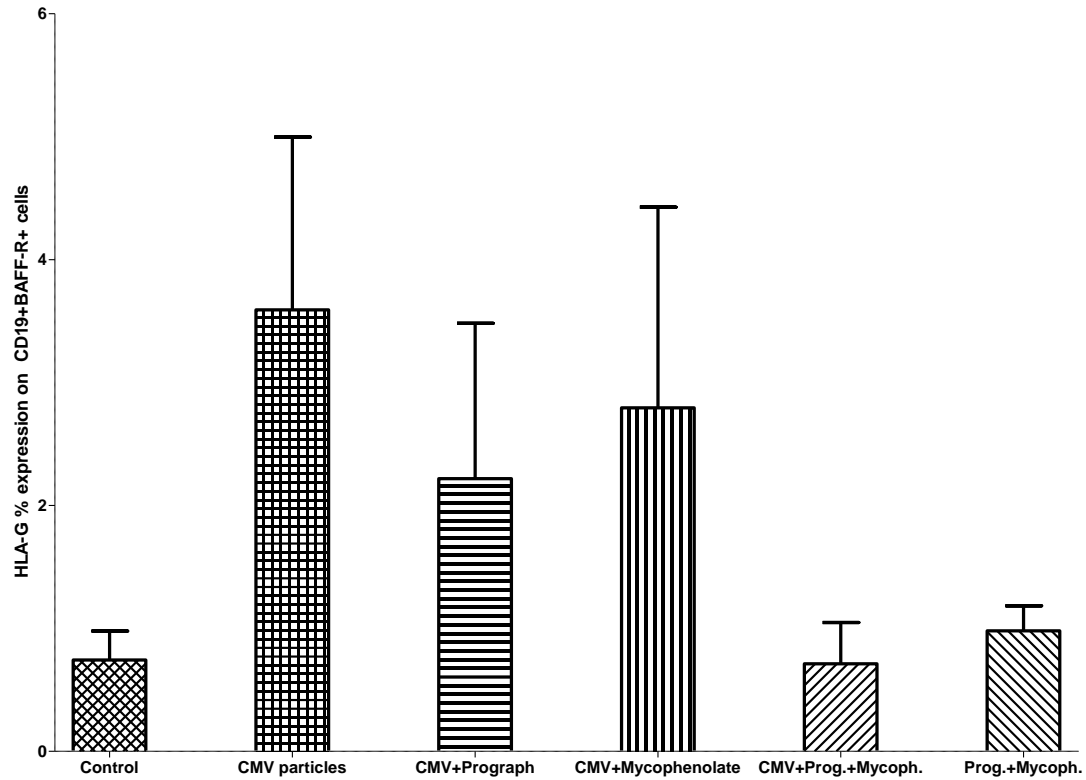


Fig. 8.39. The expression of HLA-G on CD19+BAFF-R+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.7.4. HLA-G expression by CD14+HLA-DR+BAFF-R+ cells following immunosuppressive drug treatment

Likewise, the expression of HLA-G on CD14+HLA-DR+BAFF-R+ cells was evaluated after treating the cells with immunosuppressive drugs for a week (Fig. 8.40) and as demonstrated only treatment with Prograf + Mycophenolate increased the proportion of HLA-G on these cells (4.32%) whereas the remaining treatments showed proportions of HLA-G+ cells less than the control (control 1.18%, CMV particles + Mycophenolate 0.87%, CMV + Prograf + Mycophenolate 0.13%, CMV particles 0.10% and CMV particles + Prograf 0.0%). No statistically significant difference was noted when a one-way ANOVA (Dunnett's Multiple Comparison Test) was performed ($P = 0.5437$).

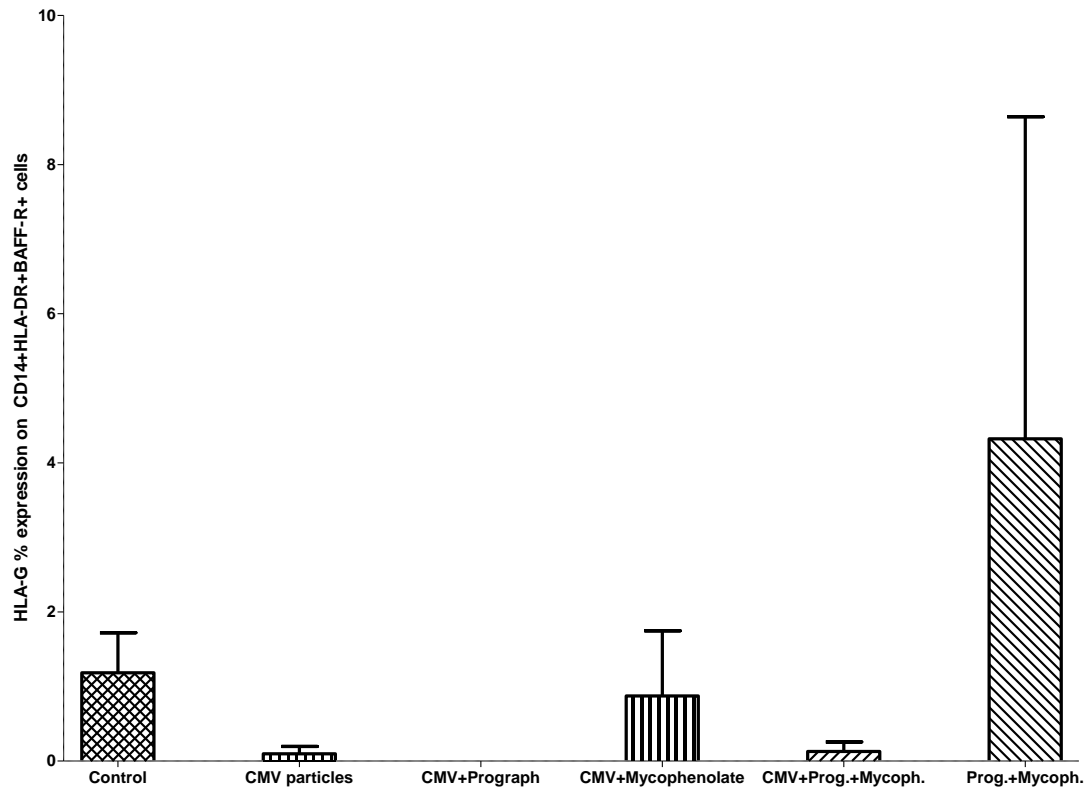


Fig. 8.40. The expression of HLA-G on CD14+HLA-DR+BAFF-R+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.7.5. Expression of HLA-G by CD3+CD56+BAFF-R+ cells following treatment with immunosuppressive drugs

Similar to above, the expression of HLA-G on CD3+CD56+BAFF-R+ was measured after culture with immunosuppressive drugs (Fig. 8.41) and there was a significant increase in the HLA-G+ proportions of these cells especially in CMV particles + Prograf 29.50% and CMV particles 29.10% treatments. The other treatments also showed increased proportions of HLA-G+ cells but to a lesser extent (CMV particles + Mycophenolate 15.12%, Prograf + Mycophenolate 13.97% and CMV + Prograf + Mycophenolate 9.51%) vs 1.16% in the control. Upon performing a one-way ANOVA (Dunnett's Multiple Comparison Test), a statistically significant difference was obtained ($P = 0.0005$), particularly significant differences were found in CMV particles + Prograf vs control ($P < 0.01$) and CMV particle vs control ($P < 0.01$).

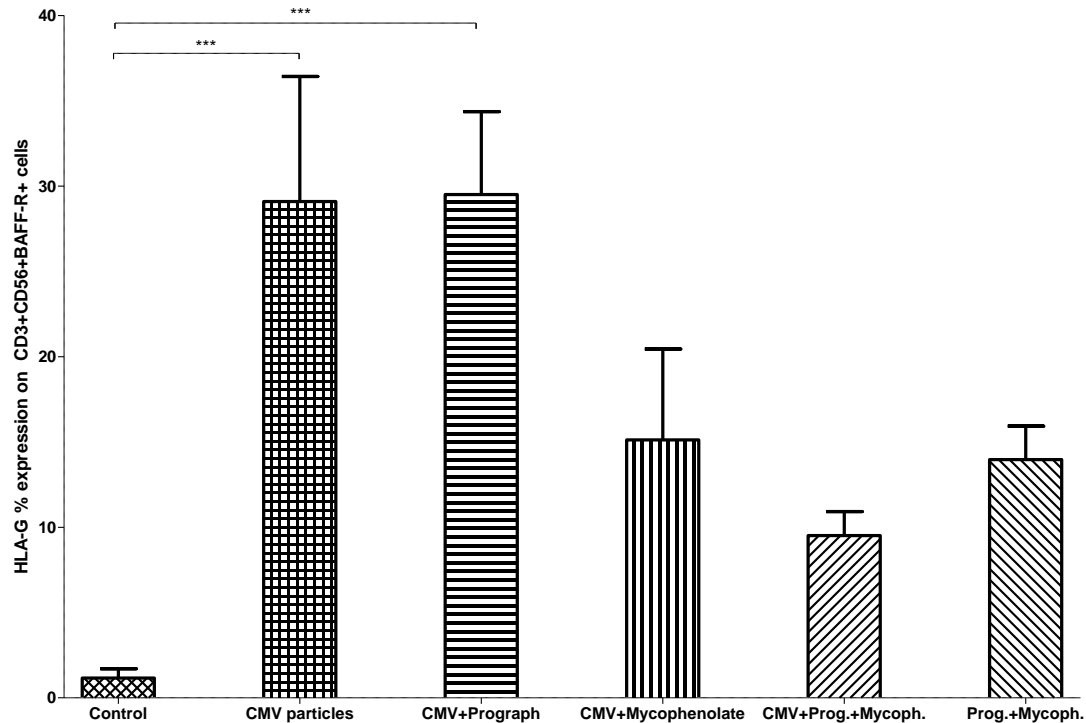


Fig. 8.41. The expression of HLA-G on CD3+CD56+BAFF-R+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, *** $P < 0.01$.

8.7.6. HLA-G expression by CD56+BAFF-R+ cells following treatment with immunosuppressive drugs

Finally, HLA-G expression was examined on CD56+BAFF-R+ cells following immunosuppressive drug treatment for 7 days (Fig. 8.42); all the treatments showed increased proportions of HLA-G+CD56+BAFF-R+ cells and the proportions in different treatments are arranged in following order; CMV particles + Prograf 7.98%, CMV particles 6.90%, CMV + Prograf + Mycophenolate 5.42%, Prograf + Mycophenolate 3.89%, CMV particles + Mycophenolate 2.84% and 0.50% in the control. No statistically significant difference was noted after performing a one-way ANOVA (Dunnett's Multiple Comparison Test; $P = 0.3226$).

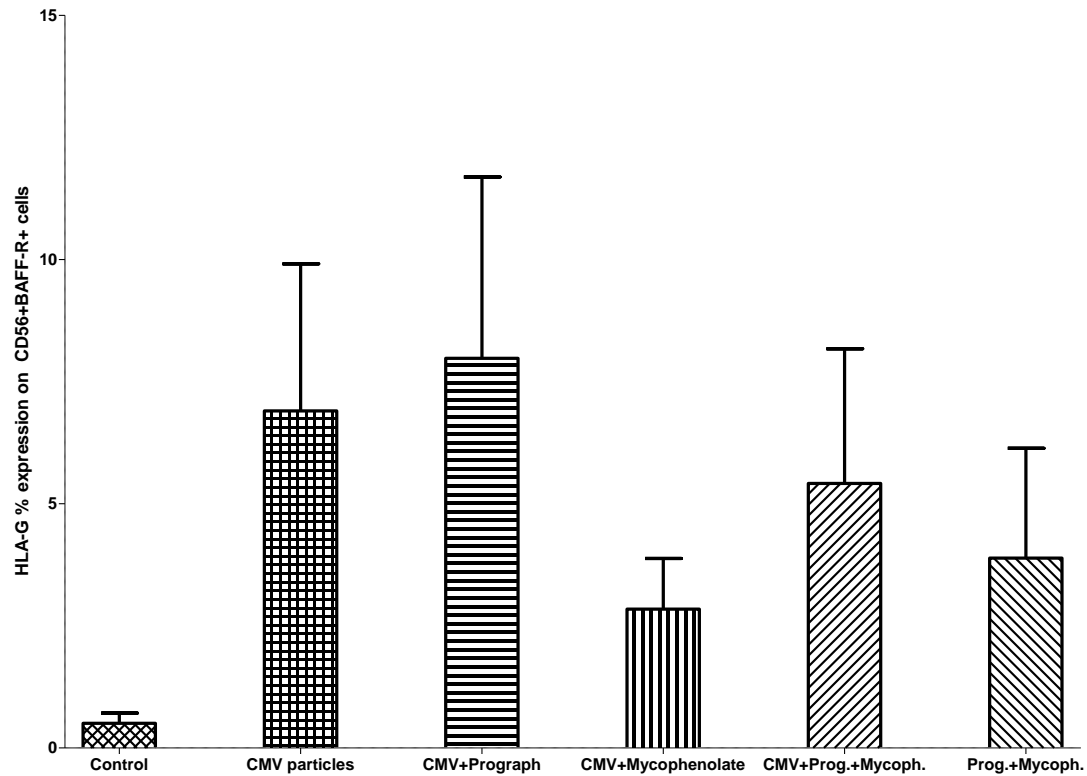


Fig. 8.42. The expression of HLA-G on CD56+BAFF-R+ lymphocytes following culture with CMV particles and immunosuppressive drugs for a week. Results are expressed as mean \pm SEM, $n = 5$, $P > 0.05$.

8.8. Summary and discussion of effects of immunosuppressive drugs on expression of HLA-G, CD103, BAFF and BAFF-R

In summary, CMV treatment generally led to increases in proportions of cells expressing HLA-G, BAFF, BAFF-R and CD103 in similar cell subpopulations to those previously reported in Chapters 3-6. The only instances of immunosuppressive drugs significantly enhancing those changes in expression were as shown in Table 8.1 where in several cases Prograf or Mycophenolate enhanced the effects of CMV to give significant increases in proportions of HLA-G+ or CD103+ cells. There were three instances where immunosuppressive drug treatments alone increased proportions of HLA-G+ cells in CD19+CD103+, CD3+CD56+CD103+ and CD56+CD103+ populations.

Figure	Molecule	Cell population	Treatment
8.1	HLA-G	CD3+CD4+CD69+ cells	CMV + Prograf
8.3	HLA-G	CD3+CD4+ cells	CMV + Mycophenolate
8.12	HLA-G	CD56+ cells	CMV + Prograf
8.19	CD103	CD3+CD56+ cells	CMV + Prograf
8.24	HLA-G	CD19+CD103+ cells	Prograf + Mycophenolate
8.25	HLA-G	CD3+CD56+CD103+ cells	Prograf + Mycophenolate
8.26	HLA-G	CD56+CD103+ cells	Prograf + Mycophenolate
8.53	HLA-G	CD3+CD8+BAFF-R+ cells	CMV + Prograf

Table 8.1. Summary of significant differences in proportions of cell subset expression of HLA-G and CD103 following immunosuppressive drug treatments.

Regarding the effects of immunosuppressive drugs on HLA-G expression, it was found that HLA-G upregulation took place on CD4+ lymphocytes, B lymphocytes and NK cell groups (CD3+CD56+ and CD56+ cells) in response to tacrolimus (Prograf) treatment. This could be due to the influence of CMV particles presented in the combination of treatment with tacrolimus because earlier the current study showed significant upregulation of HLA-GG in response to CMV induction in healthy subjects. Possibly, the upregulated HLA-G on those group of cells might be due to the medication itself because the aim of introducing immunosuppressive medication to transplant patients is to induce depletion or inhibition of T cells to allow minimal immune response against graft tissue, this could result in the expansion of other sets of lymphocytes including CD4+ Treg cells which could have potent regulatory effects on the remaining cells (Goldrath et al, 2004; Salama et al, 2003).

On the other hand, CD103 expression on peripheral blood PBMCs in response to immunosuppressive drugs was shown a pattern of upregulation on most of the assessed PBMCs, this may be attributed to the influence of CMV treatment rather than the of tacrolimus or mycophenolate induction as the it has been reported

earlier (Albayati et al, 2017), this upregulation could enhance the expression of CD103 on lymphocytes in peripheral circulation which could involve the presence of T reg lymphocytes that may express CD103 and facilitate and/or enhance epithelial receptor E-cadherin adhesion (Annacker et al., 2005; Banz et al., 2003; Lehmann et al., 2002). The upregulation of CD103 on PBMCs could participate in driving CD103+ PBMCs to infiltrate graft tissue and ligate with its ligand, E-cadherin, presented on epithelial cells, hence promoting early graft rejection (Uss et al., 2006; Wever et al., 1998). This could magnify the role of CMV in the induction of CD103 expression on PBMCs and the consequence of migration of these cells to renal tissue where they can induce early dysfunction through interaction with E-cadherin on renal epithelial cells.

Regarding the expression of BAFF protein on various PBMCs, data presented here showed no significant upregulation obtained in all treatments. This could partially due to the propagation of CMV virions which can be recognised by Toll Like Receptor (TLR-9) (Kuenzel et al., 2010) which may induce the expression of BAFF on B cells causing activation of these cells and hence antibody production (Abu-Rish et al., 2013). A possible elucidation relies on antibody mediated recognition of either self-antigen or alloantigen which could induce switching of B cells Abs from IgM to IgG (Litinskiy et al., 2002). Also, T lymphocyte activity and antibody switching were shown to be influenced by the interaction of BAFF with its ligands (Thibault-Espitia et al., 2012). The combination tacrolimus (Prograf) and Mycophenolate have not shown any significant influence on BAFF expression and CMV treatment had more impact rather than the drugs in each individual treatment group. This could indicate the potent effect imposed by CMV which may override the suppressive properties of the medications, as most of the upregulated BAFF protein observed in treated PBMCs in comparison to control and drug treatment without CMV particles could be derived from CMV upregulation as discussed earlier.

On the other hand, BAFF-R have shown no significant changes in response to immunosuppressive treatment. Thought, there was a pattern of upregulation on some PBMCs population this may be due to increase the efficiency of BAFF binding with its receptor, enhancing proliferation and improving the efficacy of these activated cells to synthesise and release immunoglobulin which may induce antibody mediated tissue rejection (Thibault-Espitia et al., 2012). In renal transplanted graft, The expression of BAFF-R in CMV+ kidney patients was recorded to be significantly

higher than in CMV- patients with elevated levels of IgG in the former group (Haiyan Xu et al., 2014). This could possibly participate to Abs mediated rejection and early dysfunction of renal graft in elevated levels of BAFF-R expression.

CHAPTER NINE

DISCUSSION

The current study was designated to evaluate the influence of CMV (AD-169 strain) protein induction on the expression of non-classical HLA-G molecules in healthy subjects to investigate the effects of HLA-G during viral infection. Also, KIR2DL4, a HLA-G ligand, was assessed in response to IL-2 induction in healthy subjects. Likewise, CD103, BAFF and BAFF-R proteins were also investigated in the study, those receptors were explored in response to CMV induction for a week in healthy individuals to check if these antigens may exert any up or down regulation during CMV infection. These cell surface molecules were chosen for investigation in later chapters as in Chapter 3 it became clear that B cells and CD56+ T cells were the cell subsets showing the greatest increase in % expression of HLA-G in response to CMV antigen stimulation. BAFF and BAFF-R are integral in B cell function and CD103 is an integrin involved in promoting lymphocyte adhesion within epithelial sites.

Additionally, the effects of immunosuppressive drugs (Prograf and Mycophenolate) were appraised to study their influence on the expression of HLA-G, CD103, BAFF and BAFF-R following treatment with different combination of the drugs in the presence and absence of CMV particles. The second part of the present study involved evaluating HLA-G and KIR2DL4 expression in renal transplant patients before and after transplantation in a sequential time frame to test whether those receptors have any effects on renal transplant outcome. The rationale for these experiments was that in Chapter 3 CMV was found to be able to induce HLA-G expression in healthy subjects; in renal transplant patients immunosuppressive drug therapy may allow CMV infection to emerge and induce HLA-G expression post transplantation. Also, these experiments were designed to test whether the immunosuppressive drugs themselves may be able to influence HLA-G expression.

Blood samples were obtained from all participants and mononuclear cells were isolated using gradient centrifugation and a major portion of PBMCs were cultured with 1µg/ml CMV proteins for seven days. The remaining PBMCs were freshly labelled with mAbs for HLA-G, CD103, BAFF and BAFF-R. Following culture, cells were recovered and labelled with the same panel of mAbs and data were acquired with Accuri C6 flow cytometry and gated as lymphocytes and monocytes based on the FSC and SSC (depending on cell size and granularity). The gated cells were then analysed in new graphs with allocated mAbs for each cell type. The proportions of HLA-G+ cells were estimated on various PBMC subpopulations including CD3+CD4+, CD3+CD8, CD19, CD14+HLA-DR+ and CD3+CD56+ and CD56+CD3-

cells. In each run, there were isotype controls acting as a cut-off for positive and negative cells, non-labelled PBMCs were also included to detect any autofluorescence. The proportions of the examined receptors were measured on various lymphocytes and monocytes and expression proportions at rest and at day 7 and data were plotted in graphs showing either resting cells, after culture or a comparison of both.

9.1. Induction of HLA-G in response to CMV proteins stimulation in healthy subjects

The current study demonstrated that HLA-G expression on various groups of freshly isolated PBMC from CMV- and CMV+ subjects is very low with average proportions being around 1% or less on most T cells, B lymphocytes and monocytes. Similar results were reported by Lozano et al who found that a very low percentage of lymphocytes and monocytes expressed HLA-G in healthy subjects (Lozano et al., 2002). Also, these results are consistent with Feger et al. who concluded that CD4+ HLA-G+ and CD8+HLA-G+ cells are circulating in peripheral blood of healthy subjects in low numbers. The HLA-G expressing cells in resting PBMCs may exhibit a monitoring role that could suppress such conditions like tumours and autoimmune disorders (U. Feger et al., 2007). In physiological circumstances the bulk of HLA-G expression is mainly limited to feto-maternal decidual tissue where HLA-G act as a tolerogenic molecule to prevent maternal immune reaction against foetal tissues (V. Rebmann, F. da Silva Nardi, B. Wagner, & P. A. Horn, 2014).

The findings of the present work have revealed that culture of PBMCs from both CMV- and CMV+ healthy subjects with CMV antigens can significantly induce the expression of HLA-G on various mononuclear cells, in particular CD3+CD56+ and CD19+ lymphocytes, however, monocytes have shown the ability to upregulate the protein following culture with CMV antigens but not to significant levels. Onno et al. have revealed that HLA-G expression can be provoked in response to HCMV infection of macrophage culture *in vitro* and no HLA-G protein was noted in the non-stimulated macrophages even after seven weeks of culture (Onno et al., 2000). On monocytes, HLA-G was induced but not to significant levels, suggesting that HLA-G expression on these cells could influence their antigen presenting functionality (Lozano et al., 2002). This was also confirmed by Yan et al, who reported a

significant elevation of HLA-G expression in monocytes and in plasma following acute CMV infection (Yan et al, 2009). Since HLA-G can induce tolerance in a variety of pathological and non-pathological circumstances, viral upregulation of this molecule can have beneficial effects on the virus by favouring immune escape (Carosella, Moreau, Lemaoult, & Rouas-Freiss, 2008). Induction of HLA-G during CMV infection can suppress the activity of a variety of cells expressing HLA-G receptors that engage with the viral clearance; NK cells have critical effects in inhibiting viral propagation and restricting its spread in active infection (Wilkinson et al., 2008). CD56+ T cells in particular have been reported to be increased in healthy CMV+ subjects and they proliferate further following CMV induction (Almehmadi et al, 2014). This is in accordance with the current study in which significantly increased proportions of CD3+CD56+ cells exhibited HLA-G expression following CMV induction suggesting the important role played by these cells in defence against CMV. However, this may also increase their immunosuppressive potential towards any other host cells expressing HLA-G ligands.

Also, the upregulation of HLA-G on other PBMC groups as depicted in Chapter 3, can be explained by the viral impact on these cells to express tolerogenic molecule that suppress cytotoxic effects of CTL and NK cells (Lin et al., 2007). However, the finding that increased proportions of HLA-G expressing CD19+ B lymphocytes as demonstrated in Chapter 3 in both CMV- and CMV+ subjects expressed the protein following CMV induction, could be due to cell to cell interaction or via signalling pathways provoked by unknown viral mechanisms which would then allow B cell to switch off other immune cells like CD4+ or CD8+ T cells. This suggestion was questioned when B cells were isolated from whole PBMCs and cultured with viral antigen with no detectable HLA-G noted. In addition, the expression of HLA-G could be influenced by post transcriptional factors like stability of mRNA and the amount of translation of the molecule (Chu et al, 1999). CMV can impose its influence via post transcriptional machinery that can modulate the activity of HLA-G genes or via other cellular or molecular pathways (Onno et al., 2000). Added to this, B lymphocytes displaying HLA-G in response to CMV induction could have potential effects on inhibiting T helper lymphocyte activity as part of the antigen presentation process. These B cells could carry a potential role in CMV infection and further studies may be required to fully understand their role in CMV infection (Albayati et al., 2017). Moreover, B lymphocytes have the ability to express an inhibitory receptor ILT-2, an HLA-G ligand, and this could be a receptor for HLA-G interaction

allowing suppression of B cells activities (Naji et al., 2014). This could potentially operate in trans fashion via other cells expressing HLA-G or in cis fashion via interaction between HLA-G and ILT-2 on the same B cell. Future work could investigate this and it would also be of interest to determine whether only CMV-specific B cells express HLA-G following CMV antigen stimulation.

9.1.1. Influence of 14bp dimorphism and SNPs in HLA-G gene on the expression of HLA-G

Regarding the HLA-G 14bp polymorphisms, the data here demonstrated no association between the HLA-G protein levels expressed by PBMCs from healthy subjects and the occurrence of the polymorphic patterns (-/-, -/+ and +/+). Previously, Hviid et al. had correlated the 14bp polymorphism in exon 8 with HLA-G protein expression (Hviid et al., 2003). This is in alignment with Jiang et al. who described elevated levels of HLA-G in hepatocellular carcinoma tissues associated with the occurrence of homozygous (-/-) but not the (+/+) polymorphism of the 14bp segment (Jiang et al., 2011). Also, Zheng et al. found that incidence of -/- alleles in HLA-G is significantly associated with active CMV infection and the number of secreted viral DNA copies is significantly higher in urine in -/- subjects than those with the +/+ polymorphism (Zheng, Zhu, Shi, Lin, & Yan, 2009b). In the same context, Chen et al. revealed that plasma sHLA-G expression is significantly lower in healthy subjects with the +/+ genotype than those with -/+ and -/- genotype (X. Y. Chen et al., 2008). However, Haddad et al. who reported no association of the 14bp polymorphism with human T lymphocyte virus 1 (HTLV-1) (Haddad et al., 2011) while, Laaribi et al. reported positive correlation of the incidence of the 14bp insertion allele with elevated levels of hepatitis B virus DNA (Laaribi et al., 2015). To the contrary, no association was found between low or high expresser HLA-G alleles and sHLA-G in French and Brazilian normal people (G. Martelli-Palomino et al., 2013). These conflicting results obtained from several groups and the present study could be due to further allelic variations in the HLA-G genes in different populations as some HLA-G alleles i.e. HLA-G*01:01:01 have higher levels of sHLA-G secreted in plasma than G*01:05 N and G*01:01:03, while the former alleles express less sHLA-G than G*01:04:01 (Rebmann et al., 2001). The occurrence of the 14bp insertion polymorphism in exon 8 of HLA-G could lead to less sHLA-G expression in sera due to lower mRNA quantities (Hviid et al., 2003;

Hviid et al., 2004). Also, the experiments carried out here utilised antigens from a laboratory CMV strain (AD169) to stimulate PBMCs and these viral antigens are missing a few gene products found in clinical strains (Cha et al., 1996); as such, it is suggested that laboratory CMV strains do not fully mimic clinical isolates (Albayati et al., 2017).

Similarly, data presented in this thesis revealed no clear association between most of the SNPs and the levels of HLA-G+ cells after CMV culture, however, only C/A+3027 and C/T +3035 were positively correlated with increased proportions of HLA-G+ cells following CMV induction. Tan et al. observed an association between the incidence of the C allele and the expression of HLA-G in patients susceptible to asthma (Tan et al., 2007). Castelli et al. have confirmed the influence of the C +3142 SNP on microRNA binding, the occurrence of this SNP could affect microRNA binding which could be translated into more HLA-G production (Castelli et al., 2009). This was consistent with Martelli-Palomino et al. who reported increased levels of sHLA-G with the incidence of C/G +3035 SNP (Gustavo Martelli-Palomino et al., 2013) while another group have shown the association of the G +3187 SNP with pre-eclampsia patients (Quach, Grover, Kenigsberg, & Librach, 2014), although HLA-G levels were not measured in this study.

Complex processing involved in the synthesis of HLA-G may be influenced by the promoter region, degradation and transcription of mRNA, also other polymorphisms in HLA-G encoding regions can influence the levels of mRNA translation and consequently the amount of HLA-G expression (E. A. Donadi et al., 2011). The presence of the SNPs in 3'UTR of HLA-G could have potentially affect the affinity of microRNA as well as the mRNA stability, this could be noted especially with G/C +3142 and A/G +3187 SNPs in the presence of the 14bp insertion variant (Castelli et al., 2009; E. A. Donadi et al., 2011). In addition, microRNA binding positions and affinity could be affected by the occurrence of other SNPs like C/T+3003, G/C +3010, and C/A +3027 (Castelli et al., 2009). The controversial results of the SNPs influence on HLA-G expression could be also attributed to the small sample sizes in the present study or the sampling of DNA as it has been obtained from different ethnic groups.

9.1.2. sHLA-G concentrations in healthy subjects

This study reported no significant differences in sHLA-G concentration in plasma from CMV- and CMV+ individuals as well as from supernatant recovered from CMV-PBMCs culture induced with and without CMV proteins while, supernatant obtained from CMV+ subjects stimulated with or without CMV antigens had a significant increase in sHLA-G after induction. In CMV+ subjects the increased secretion of sHLA-G in response to CMV induction could indicate the possibility of this protein to interact with receptors for HLA-G like KIR2DL4, ILT-2 and ILT-4 that mediate inhibitory behaviour of some immune cells in the presence of CMV infection (Albayati et al., 2017). Rizzo et al. reported a significant increase of sHLA-G in serum samples from primary CMV+ pregnant women compared to CMV- and CMV carrier control (Rizzo et al., 2016). Similarly, in Hepatitis B virus infection and chronic hepatitis C virus infection sHLA-G in sera was elevated in both conditions compared to control healthy volunteers (Shi et al., 2011; Weng et al., 2011). Also, there were significantly elevated sHLA-G concentrations in plasma noted in HIV infected patients which decreased after ART drug treatment (Murdaca et al., 2009). However, these results differ from those in the current study, mainly due to the fact that those healthy subjects involved in the assessment of sHLA-G were not having acute CMV infection or reactivation of CMV at the time of blood sampling.

sHLA-G could potentially suppress T lymphocyte alloproliferative activity (Lila, Rouas-Freiss, Dausset, Carpentier, & Carosella, 2001). Also, sHLA-G has the ability to interact with one of its ligands, KIR2DL4, found on NK cells and other PBMCs can mediate inflammatory mediator synthesis (van der Meer et al., 2007). In addition, the binding of sHLA-G with ILT-4 on DC can activate T reg which in turn participate in induction of tolerance favouring CMV evasion (LeMaoult, Krawice-Radanne, Dausset, & Carosella, 2004). The induced sHLA-G demonstrated in supernatants from CMV+ subjects could be due to the influence of CMV as a strategy by which the virus can escape the effects of NK cells. This suggestion is in line with that of Spaggiari et al. who reported the ability of sHLA-G to provoke self-killing in NK cells via the activation of the Fas/FasL pathway when CD8 antigen interact with sHLA-G molecules (Spaggiari et al., 2002).

9.2. KIR2DL4 expression in healthy subjects

The data presented in this research have manifested that there was a significant increase in the expression of KIR2DL4 in CD3+CD56+ lymphocytes and significant downregulation of the protein on CD56^{bright} cells following IL-2 induction. However, a base line expression was noted in resting PBMCs. These data are not compatible with observations made by other groups who reported no cell surface KIR2DL4 expression in naïve NK cells extracted from whole blood (J. P. Goodridge et al., 2003; Kikuchi-Maki et al., 2003). This could be due to the protein being an intracellular receptor residing in endosomes (Rajagopalan et al., 2006) or that the mAb clone used did not recognise the cell surface receptor. The expression of this receptor on cell surface of some resting PBMCs may suggest that this molecule may act as a co-receptor that co-exists with other ligands like ILT-2 which is exhibited on NK cells, T lymphocytes and monocytes (Colonna et al., 1997). While, Kikuchi-Maki et al. and Rajagopalan et al. have revealed that KIR2DL4 could be induced on the surface of NK cells following IL-2 induction (Kikuchi-Maki et al., 2003; Rajagopalan et al., 2006), the data presented in the current study are in agreement with Rajagopalan & Long who described the ability of this protein to be expressed on NK cell surface of virtually all healthy individuals (S. Rajagopalan & E. O. Long, 1999) and its expression is limited to CD56^{bright} cells in peripheral blood (Kikuchi-Maki et al., 2003). Resting T cells and NK cells expressing this receptor could be as a part of tolerogenic strategy by this molecule upon interacting with its ligand, HLA-G, as the fusion of KIR2DL4 with HLA-G takes place within endosomes and can result in the modification of cytokines released by NK cells (Rajagopalan et al., 2006; Sumati Rajagopalan & Eric O. Long, 1999).

This sort of interaction was reported to happen in decidual NK cells during pregnancy which can maximise tolerance of foetal tissues by maternal immune cells (Ponte et al., 1999). Albeit, the cytotoxic function of NK cells could be declining when this protein binds with its ligand in cell surface interaction and following induction with IL-2 in vitro which could also result in downregulating its expression on the NK cell surface (J. P. Goodridge et al., 2003; Kikuchi-Maki et al., 2003). The interaction of KIR2DL4 with soluble or cell-associated HLA-G could give rise to proinflammatory cytokine release by NK cells with inhibition of cytotoxic activity (Kikuchi-Maki et al., 2003; Rajagopalan et al., 2006; Yu, Tian, Wang, & Feng, 2006). The dual behaviour of KIR2DL4 upon interaction with HLA-G could be

attributed to the presence of two domains, a cytoplasmic suppressing region and a transmembrane activating tail (Vera Rebmann, Fabiola da Silva Nardi, Bettina Wagner, & Peter A. Horn, 2014).

The current results have demonstrated that the expression of KIR2DL4 was not influenced by the KIR2DL4 9A and 10A genotype as the receptor was found on the cell surface in both 9A and 10A variants. Previously, it has been reported that KIR2DL4 expression is influenced by some alleles (J. P. Goodridge et al., 2003) and occurrence of the 10A allele of the KIR2DL4 gene encodes the full length protein, unlike the 9A variant (Goodridge et al., 2007). This would give rise to frame shifting after the 9A sequence which can result in generation of a stop codon in exon 7 affecting the cytoplasmic domain, consequently, the translation of this gene may not produce expression or an unstable protein will be expressed on the cell surface (Witt, Martin, & Christiansen, 2000). However, the presence of the 10A allele could have some effects on the role of KIR2DL4 by the activating function of the protein being dominant (Goodridge et al., 2007).

9.3. CD103 expression in healthy individuals

The findings of this study regarding CD103 expression by peripheral blood PBMCs from healthy subjects demonstrated very low levels of this receptor on resting cells but following induction with CMV antigens, there was a significant upregulation of the protein by CD8+ T cells and NK cells. These findings are in consistent with those of Hadley et al. who reported the expression of CD103 on the surface of T cytotoxic cells (Hadley, T Bartlett, Via, A Rostapshova, & Moainie, 1997) and in another study this protein was shown to be upregulated in effector CD8 lymphocytes in renal transplant rejection (Robertson et al., 2001). The expression of CD103 has been shown to be associated in localising regulatory memory T lymphocytes in salivary glands in murine CMV infected mice (C. J. Smith, Caldeira-Dantas, Turula, & Snyder, 2015). CMV can potentially induce the secretion of TGF- β 1 as confirmed in the current study with qPCR, also Michelson et al. have demonstrated the upregulation of TGF- β 1 in CMV infected fibroblasts (Michelson et al., 1994a). TGF- β on the other hand can upregulate the expression of CD103 (D. Wang et al., 2004) in PBMCs which leads to induction of CD4+ T reg that have suppressive properties (W. Chen et al., 2003). In addition, CD103 may play a key role in provoking the

migration of CD8+ lymphocytes to mucosal tissues (Laidlaw et al., 2014). This suggests the influence of CD103 on mobilising such regulatory cells and CTL to the CMV active mucosal tissues where such cells can impose immunosuppressive conditions, favouring viral replication and spread.

The data presented in this study have illustrated the upregulation of HLA-G on CD103+ PBMCs after CMV antigen culture. It has been reported that CMV can influence the expression HLA-G in various tissues and immune cells as discussed earlier (Albayati et al., 2017; Rizzo et al., 2016). While, CD103 expression demands the involvement of TGF- β and TCR (Allakhverdi et al., 2006; P. W. Robinson, Green, Carter, Coadwell, & Kilshaw, 2001) these elements can be induced during the course of CMV infection which can favour the immunosuppressive influence of these molecules to allow CMV evasion of the immune response and persistence. Moreover, the cells expressing CD103 and HLA-G could be regulatory cells or even effectors necessary in immune regulation (Allakhverdi et al., 2006; Laidlaw et al., 2014).

9.4. BAFF and BAFF-R expression in healthy subjects

Regarding BAFF antigen, this protein was shown to be slightly increased in response to CMV induction after a week of incubation, although no significant differences were recorded compared to the cultured control cells. This could illustrate the effects of CMV in upregulating this molecule on different cell populations in order to induce a tolerogenic microenvironment that may participate in the process of viral escape from the impact of effector immune cells. It has been found that the expression of BAFF and sBAFF were dramatically elevated in CMV positive renal transplant patients, suggesting a positive association of CMV and BAFF protein (Haiyan Xu et al., 2014). This receptor could be upregulated by CMV during the course of infection via two possible mechanisms; the first one suggests that CMV infection is associated with virion production which are identified by Toll Like Receptor-9 (Kuenzel et al., 2010); this receptor when activated by CMV virions could result in enhancement of BAFF synthesis by PBMCs (Abu-Rish, Amrani, & Browning, 2013). This could effectively increase antibody production and possibly induce switching of Ab classes (Litinskiy et al., 2002). The second mechanism suggests the induction of BAFF through an INF mediated pathway, as it has been shown that CMV can potentially upregulate INF- γ and - α secretion (Netterwald et

al., 2004) which in turn could promote BAFF expression (Ittah et al., 2006). This was also consistent with the qPCR findings reported in the current study which emphasise significant upregulation of INF- γ in B cells isolated from whole PBMCs following culture with CMV antigen. This was also in line with Ittah et al. who infer augmented mRNA levels in epithelial cells in response to INF- γ and INF- α induction (Ittah et al., 2006). This may induce cytokine production and/or a BAFF mediated tolerance mechanism that spare viral recognition by immune cells.

Data presented in this research indicated significant expression of HLA-G on BAFF+ PBMCs. A rationale for this may involve the upregulation of TGF- β as a consequence of CMV signalling (M. Shimamura, J. E. Murphy-Ullrich, & W. J. Britt, 2010) which could in turn promote the induction of HLA-G on circulating PBMCs (Guan et al., 2015). More potent immunosuppressant HLA-G+BAFF+ cells could further hinder the immune action against CMV infection, favouring viral survival.

On the other hand, the data presented in this work have revealed significant down regulation of BAFF-R on CD19+ cells and significant upregulation of this receptor on CD56+ cells. BAFF-R can also be expressed on T lymphocytes including T reg in addition to its abundant expression on B cells (Ye et al., 2004). This receptor plays a key role in B cell maintenance and propagation and is considered the sole ligand for BAFF protein (Y.-J. Woo et al., 2011). A probable explanation for this receptor upregulation involves the induction of the NF- κ B pathway under the influence of CMV infection (DeMeritt, Milford, & Yurochko, 2004) which could potentially promote the expression of BAFF-R on PBMCs, as Woo et al. demonstrated that downregulation of NF- κ B could potentially suppress the expression of BAFF-R on PBMCs (Woo et al., 2011). BAFF-R expression on B lymphocytes could have negative effects on CMV and activation of this receptor could involve BAFF expression thus improving B cell activity and defensive properties (Thibault-Espitia, Foucher, Danger, Migone, Pallier, Castagnet, G.-Gueguen, et al., 2012). This may play role in viral suppression via an antibody mediated response which ultimately leads to viral clearance. However, BAFF-R expression was inhibited on B cells as indicated earlier in the results which suggests a viral role in suppressing this molecule.

As far as HLA-G expression on BAFF-R+ PBMCs is concerned, it was found that a significant proportion of those BAFF-R+ cells co-expressed HLA-G. This suggests the important role played by HLA-G to be displayed on such cell populations to enhance

viral replication and to allow immune escape. Since upregulation of NF- κ B could essentially be achieved by viral signalling, NF- κ B is capable of controlling the secretion of INF- γ and IL-2 and IL-12 by T helper cells (Y. J. Woo et al., 2011) together with TGF- β , this could promote HLA-G expression on PBMCs (Guan et al., 2015; Michelson et al., 1994a).

sBAFF levels on the other hand did not differ in serum from CMV+ or CMV- healthy subjects; in addition, supernatants from CMV induced and non-induced PBMCs have also revealed no significant changes. This may be attributed to the inhibition of BAFF-R noted on CD19+ cells as described earlier, which could inhibit the production of this protein in supernatants. Also, CMV status had no influence on the levels of sBAFF indicating no recurrence or reactivation of CMV because all the participants were healthy with no illness reported. sBAFF has been reported to be augmented in CMV+ kidney transplant patients (Haiyan Xu et al., 2014) and an increase in its concentration was found to correlate with alloantibody synthesis (Thibault-Espitia, Foucher, Danger, Migone, Pallier, Castagnet, C, et al., 2012) which was not reported here.

9.5. Cytokine gene expression

Gene expression experiments presented in the current study demonstrated a significant increment of gene expression of some cytokines (TGF- β , INF- γ , and TNF- α) in response to CMV induction in B cells isolated from whole PBMCs culture while the remaining cytokines (IL-6 and IL-8) were not significantly upregulated. These findings are in line with previous reports which indicated the upregulation of TGF- β in response to CMV infection (Michelson et al., 1994b; Masako Shimamura, Joanne E. Murphy-Ullrich, & William J. Britt, 2010). Also, gene expression of this cytokine could be potentially increased during CMV infection (Masako Shimamura et al., 2010). A suggested mechanism by which TGF- β could be promoted involves some activators like plasmin, integrins α v β 6 and - β 8 and matrix metalloproteases (MMP) (Annes, Munger, & Rifkin, 2003; Masako Shimamura et al., 2010). TGF- β could have a prominent impact on inhibiting cellular proliferation as well as on the response of the immune cells (Michelson et al., 1994a).

Similarly, elevated level of INF- γ gene expression was found in induced cells with CMV antigens, this could be attributed to the possibility of stimulated T cells when

engaging with viral antigens are promote to secrete INF- γ as part of an antiviral strategy (van den Pol et al., 2007). This could lower the infectivity of CMV and inhibits viral induce cellular damage. From a viral point of view, INF- γ can inhibit the expression of classical MHC I molecules on the cell surface (Halenius et al., 2011), allowing viral induced HLA-G to be expressed proportionately more highly, thus creating a tolerogenic microenvironment favouring surveillance escape.

Likewise, TNF- α gene expression was substantially augmented in CMV treated cells, this is in line with previous studies which demonstrated the impact of CMV on increasing levels of TNF- α (Geist, Monick, Stinski, & Hunninghake, 1994; P. D. Smith, Saini, Raffeld, Manischewitz, & Wahl, 1992; Turtinen, Assimacopoulos, & Haase, 1989). Probably this could be due to the effects of immediate early genes of CMV which could increase mRNA promoter activity causing elevation in gene expression and consequently more cytokine translation and release (Geist et al., 1994). This could be implicated in maintenance of viral activity and propagation of new virions, as it has been shown that TNF- α is crucial for triggering immediate early genes of CMV which can be achieved through the NF- κ B signalling pathway (Simon, Seckert, Dreis, Reddehase, & Grzimek, 2005).

As far as IL-6 gene expression is concerned, this cytokine did not show significant elevation following CMV induction, but it was upregulated compared to control non-stimulated cells which could suggest that this cytokine may play role in CMV pathogenesis. Some studies have illustrated the upregulation of this cytokine during CMV infection (Carlquist, Edelman, Bennion, & Anderson, 1999). The rationale behind IL-6 induction in CMV induced cells may be explained through the induction of NF- κ B by viral immediate early genes which could upregulate IL-6 production (Carlquist et al., 1999). This cytokine has an important mediator role in inflammation with a range of activities involving lymphocyte propagation and differentiation as well as suppressing apoptosis (Kamimura, Ishihara, & Hirano, 2003; Moreno et al., 2001; Roca et al., 2009). This may suggest the ability of CMV to exploit the anti-apoptosis property offered by IL-6 to evade recognition by immune effector cells.

With respect to IL-8, the data shown in this study demonstrated increase in gene expression of this cytokine but not to a significant level. Other groups have described an elevation of IL-8 in CMV infection (Alcendor, Charest, Zhu, Vigil, & Knobel, 2012; Craigen et al., 1997; Murayama et al., 2000). The postulated mode

of IL-8 upregulation is mediated via CMV early intermediate genes which potentially could expedite activation of the NF- κ B pathway that can induce IL-8 expression (Murayama et al., 2000). So, this cytokine seems to have a pivotal role in CMV propagation as well as spread (Costa, Nascimento, Sinclair, & Parkhouse, 2013; Murayama et al., 1994) through suppressing the activity of INF- α generated by the immune cells as a counteracting measure (Murayama, Mukaida, Khabar, & Matsushima, 1998).

Altogether, cytokines induced by CMV influence in PBMCs seems to play a crucial role in immune evasion and possibly pathogenesis of the virus and their upregulation may elucidate some strategies of CMV virulence elements to override host surveillance.

9.6. HLA-G expression in renal transplant patients

In renal transplant patients, the proportions of HLA-G-expressing cells showed slight elevation in most of the cells with significant increases noted in CD4⁺ lymphocytes, CD19⁺ cells and in monocytes after kidney transplantation. This is consistent with Lu et al. who reported a gradual increase in HLA-G expression on CD4⁺ cells following renal transplantation compared to the pre transplant levels (Lu et al., 2011). Similarly, Xiao et al. reported low levels of expression of HLA-G on CD4⁺ and CD8⁺ cells in peripheral PBMCs from renal allograft recipients (Le Rond et al., 2004; Xiao et al., 2013). A possible explanation behind these results is that activation of T and B cells or even monocytes following transplantation could be the result of tissue sensitization or mismatch in HLA antigens (Lu et al., 2011). In the current study, all patients who underwent renal transplantation were either treated with Prograf or Mycophenolate Mofetil as immunosuppressive drugs according to the protocol applied by the providing hospital. These medications could have indirect influence on HLA-G expression via increasing cytokine production like IL-12, -10, and IL-4 by activated T helper cells (Daniel et al., 2005), the expression of HLA-G can be preferentially provoked by these cytokine in monocytes and trophoblast (Moreau et al., 1999). Thus, the upregulated expression of HLA-G on PBMCs could be due to immunosuppressive effects or an ongoing immune tolerance process that is not fully clear. Consequently, the expression of HLA-G on peripheral PBMCs could enhance the chances of graft survival by delaying early rejection and suppressing

proliferating cells involved with allograft rejection (Lu et al., 2011) and this explanation is in line with the current study results in which all but one of the transplanted patients did not have acute rejection episodes. This was further supported by Feger et al. who reported the presence of a group of CD4+ and CD8+ lymphocytes in circulation that display the HLA-G molecule, these lymphocytes are potentially immune suppressor cells that have low proration rate (Ute Feger et al., 2007). Moreover, another group has noted that patients with acute graft rejection have decreased levels of HLA-G mRNA in circulating PBMCs compared to transplant patients with no rejection incidences (Racca et al., 2009). Additionally, T cell activation occurs prior to early rejection and tissue damage in cellular rejection, therefore, a decline in the expression levels of HLA-G in peripheral blood cells could antecede deteriorations in renal function parameter, so, HLA-G could be considered as biomarker for early graft rejection (Lu et al., 2011). Similar evidence has been reported by Qiu et al. who correlated decreased levels of antibodies to HLA molecules with elevated expression of sHLA-G (Qiu et al., 2006). Also, there has been significant association between high HLA-G expression and decline in early and late rejection incidence (Creput, Durrbach, et al., 2003). A possible mechanism by which HLA-G could exert its influence on effector immune cells is via suppressing CTL and NK cells cytotoxic activities and/or by inhibiting alloproliferation of helper T cells (Carosella et al., 2003). In addition, modulation of helper T cell 1 and 2 ratios may occur under the influence of cytokines released by HLA-G expressing cells that lead to higher T helper 2 cytokines producing lymphocytes (Kanai et al., 2001). All together or solely, graft rejection may be delayed or diminished. This accumulative evidence could explain the tolerogenic nature of HLA-G expressing cells in renal transplant patients and their role in minimising cell mediated and antibody induce graft rejection (Lazarte, Tumiaty, Rao, & Delgado, 2016).

9.6.1. Influence of HLA-G genotype on HLA-G expression in renal transplant patients

The influence of the 14bp ins/del dimorphism in the HLA-G gene on expression levels of HLA-G in renal transplant patients involved in the present study was investigated and no significant correlation was found between genotype and expression levels. Also, no association was identified between the incidence of the 14bp dimorphism with CMV viraemia following transplantation nor with rejection

episodes. These data are in line with Xiao et al. who recorded no association between membrane bound HLA-G and early rejection in renal and liver transplant patients (Xiao et al., 2013).

These results were consistent with those of Waterhouse et al. who described no influences of the 14bp dimorphism on rejection episodes and on sHLA-G titre in patients with haematopoietic stem cell transplantation (Waterhouse, Duque-Afonso, Wäsch, Bertz, & Finke, 2013). Similarly, Aghdaie et al. and Crispim et al. reported no effects of the 14bp dimorphism on the occurrence of early rejection in kidney transplant patients (Aghdaie et al., 2011; Crispim et al., 2008), unlike, Littera et al. who recorded significant association of the 14bp (-/-) variant with kidney malfunction and late graft rejection (Littera et al., 2013). Also, there was an increased incidence of early rejection in kidney grafts observed in patients with the 14bp (+/+) variant in comparison to patients with no rejection episodes (Z. K. Jin et al., 2012). These conflicting reports may arise from genetic variation in ethnic groups and other microenvironmental elements (Eduardo A. Donadi et al., 2011) could have different influences on HLA-G gene expression, also in the current study, relatively small numbers of transplant patients were involved which may not reflect a powerful statistical sampling, a larger sample size may produce a different outcome. Previously, Hviid et al. reported the association of the 14bp (+/+) variant with decreased mRNA levels and hence low translation of HLA-G protein (Hviid et al., 2003; Hviid et al., 2004). However, various elements could influence the translation of this protein (Moreau, Flajollet, & Carosella, 2009) like the SNP alterations occurring in the promoter region and the 3'UTR which could affect the levels of mRNA and hence levels of protein displayed on cells. Over and above that, the incidence of SNPs in intron 1-5 could exert regulatory functions affecting the splicing of the molecule (Donadi et al., 2011). Additionally, the insertion variant of 14bp is frequently associated with other SNPs like G +3142 and A +3187 SNPs and those combinations are considered as low HLA-G expressers because they have been associated with decreased mRNA synthesis (Hviid et al., 2003). Added to this, the occurrence of the 14 bp insertion generates an additional splice site leading to truncation of 92 base pairs from exon 8 of the complete mRNA form of HLA-G resulting in a shorter transcript which is even less stable than the fully mature copy (Rousseau et al., 2003). Yet, the splicing in the mRNA could be elicited from linkage disequilibrium of the 14bp ins/del with another set of polymeric sites (Donadi et al., 2011). Overall, the 14 bp polymorphism may influence membrane and sHLA-G

expression in transplant patients but the result presented in the current study did not support previous reports.

Similarly, no association was found between the frequency of the SNPs in exon 8 of HLA-G gene and the levels of HLA-G expression. These findings are not in line with those of Cilio Alves et al. who reported significant association of G/A +3187 SNP with incidence of early and late renal graft rejection, also they noted the occurrence of the +3035C SNP with delayed rejection episodes (Cilio Alves et al., 2012). Likewise, the frequency of the +3187A SNP was correlated with reduced levels of HLA-G expression *in vitro*; these could influence the mRNA levels that code for protein expression (Yie et al., 2008). Additionally, other influential factors may play a role in modifying the amount of HLA-G production for instance, the occurrence of C/G +3142 SNP could potentially affect the microRNA binding sites resulting in low mRNA levels and low protein production (Veit & Chies, 2009; Zhu et al., 2010). The miRNA binding capacity could essentially be affected by other SNPs in HLA-G 3'UTR (Castelli et al., 2009) which could either up or down regulate the synthesis of HLA-G proteins. These suggestions have been investigated in a Canadian population and there was positive correlation of incidence of the +3187A SNP with pre-eclampsia (Yie et al., 2008) as well as with Brazilian SLE subjects (Lucena-Silva et al., 2013). Additionally, protein expression may be influenced by alleles that are associated with low HLA-G synthesis like G*01:01:02, G*01:01:01:03 and G*01:06 which have been reported to be correlated with high susceptibility to rejection episodes in renal transplantation (Misra et al., 2014). Probably, the influence of the SNPs could be better elucidated in a larger group of patients as there was a relatively small group of renal transplant recipients involved in the current study.

9.6.2 sHLA-G in renal transplant patients

Serum sHLA-G in renal transplant patients was assessed and there was no significant difference in levels before and after transplantation, however, base line levels were high pre transplant and gradually increased. These findings contradict those of Qiu et al. who detected no sHLA-G in serum of pre-renal transplant samples, while those patients with rejection had significantly lower levels compared to functioning kidney recipients (Qiu et al., 2006). Likewise, another group recorded elevated levels of sHLA-G in kidney recipients in comparison to a control cohort, and

the concentration of this protein in sera was low in patients developed rejection. Also the same group reported relatively high titre of sHLA-G in recipients with normal functioning graft compared to those who experienced rejection episodes after transplantation (Tyagi et al, 2017). Similar findings were obtained by Jin et al who demonstrated elevation in sHLA-G after 3 months of kidney transplantation with a low incidence of rejection episode in those recipients (H. L. Jin et al., 2012). The data noted in the current study indicate that sHLA-G levels were high at pre transplant procedure which could be a result of the immunosuppressive drug administration applied in the protocol (Farid et al, 2015; Luque et al., 2006). Also, steroids have been reported to induce the production of sHLA-G (Levitsky et al., 2009; Moreau et al., 2001). sHLA-G could have pronounced effects on suppressing the humoral immune response towards HLA molecules (Qiu et al., 2006) through suppressing alloreactive immune responses of CD4+ lymphocytes (Lila et al., 2001) that may be reflected by impeding the activity of B lymphocytes (Lumsden et al, 2003). Beside, sHLA-G can have systemic effects on immunity through inhibiting the function of effector cells like NK cells and CTL that are associated with graft rejection by provoking apoptosis (Contini et al., 2003; Creput, Durrbach, et al., 2003). However, sHLA-G assays conducted in this study were on quite a small number of transplant patients and a larger group may produce more significant findings. Altogether, elevated sHLA-G levels as seen in the cohort involved in the present study could be a marker of better renal graft survival and an early indicator for graft acceptance as only one of the patients included in the trial had an early rejection episode.

9.7. KIR2DL4 expression in renal transplant patients

Data from KIR2DL4 revealed a trend toward increasing expression of this protein following renal transplantation on PBMCs, however, no significant expression was noted apart from on CD56^{dim} NK cells. It has been reported that NK cells express an inhibitory protein, KIR2DL4, whose ligand is HLA-G (Yan & Fan, 2005). Since the expression of HLA-G in transplant patients involved in the present study cohort was noted pre and post transplantation, the influence of HLA-G expression can be exerted via over expression of HLA-G ligand proteins like KIR2DL4, ILT2 and ILT4 especially in NK cells, APCs, and other T lymphocytes (Vera Rebmann et al., 2014). As KIR2DL4 expression was recorded on CTL, CD4+ lymphocytes and $\gamma\delta$ T cells

(Bjorkstrom et al., 2012), these cells may be subject to inhibition that may enhance the tolerability toward graft tissue and hence improve graft survival and functionality (Rouas-Freiss, Naji, Durrbach, & Carosella, 2007).

The elevated expression of KIR2DL4 after transplantation suggests an on-going process of immune sensitisation that could be leading to graft complications on the long term; Vampa et al. have reported increased NK cytotoxic activity following transplantation (Vampa et al., 2003). Also, the count of NK cells was reported to be increased in the peripheral circulation in patients with early kidney graft rejection (Cooksey, Robins, & Blamey, 1984). However, the data shown here indicate no rejection episodes in the tested group apart from one. This could be justified as the follow up period of transplanted patients was limited only to 6 months and if longer follow up period was applicable then different outcomes may be obtained. Also, current immunosuppressive treatments used in the induction protocol could be more effective than in those used in earlier studies. Overall, the expression of KIR2DL4 was clearly higher in the post transplant period suggesting an inhibitory role played by this receptor to maintain a well-functioning graft by inhibiting effector cells involved in tissue rejection.

On the other hand, KIR2DL4 genotyping has been reported to be influenced by the presence of 9A or 10A alleles in exon 6 of KIR2DL4 gene, in which 10A alleles have high protein expression on cell surface unlike the 9A allele (Goodridge et al, 2003). However, the data presented in the current study have revealed that KIR2DL4 expression is not directly influenced by genotyping as renal transplant patients with 9A and 10A alleles have been shown to express this receptor at the cell surface in relatively similar proportions. Almost all NK cells have been shown abundant levels of KIR2DL4 mRNA expression (Husain et al, 2002) and this receptor has been reported to be displayed by all circulating NK cells (Rajagopalan & Long, 1999). Goodridge et al. have demonstrated that the 10A allele has the ability to display abundant amount of the protein in PBMCs in particular on CD56^{bright} cells. These controversial results could be attributed to the clone of mAbs used in their experiments which differ from the one used in the current study. In addition, the patients in this study were renal transplanted and all of them had been receiving immunosuppressive drugs which could influence the receptor expression directly or indirectly through the upregulation of HLA-G which may in turn lead to upregulation of its ligands including KIR2DL4 (Akhter et al., 2012; Rebmann et al., 2014).

Another probable explanation is that the 9A genotype is cable of producing low levels of mRNA which could be translated to protein that lacks some properties such as the inhibitory tail (Goodridge et al., 2003; Lu et al., 2011).

9.8. Immunosuppressive drug effects on HLA-G expression

Regarding immunosuppressive drug influence on HLA-G expression, there was significant upregulation of HLA-G expression on the main lymphocytes subsets including CD4+ lymphocytes, B lymphocytes and NK cell groups (CD3+CD56+ and CD56+ cells) in response to tacrolimus (Prograf) treatment at physiological concentration in the presence of HCMV particles AD169 strain, while the remaining treatment combinations showed no significant changes and only slight elevation of HLA-G expression was recorded. HLA-G expression on these cells could be upregulated in response to CMV particles presented in combination with tacrolimus treatment, as the current study reported the ability of CMV to induce HLA-G expression on peripheral blood PBMCs from healthy subjects. Other studies have also demonstrated the capability of CMV to induce expression of HLA-G on certain PBMC subsets like monocytes, CD4+ cells and NK cells (Albayati et al., 2017; Bi et al., 2017; Onno et al., 2000). However, the mechanism by which HLA-G is expressed on some PBMC subsets is not fully clear and a possibility of cytokine mediated and/or B cells T cells interaction may play a role in provoking the expression of this protein and thus suppressing T cell activation and function (Wiseman, 2016).

On the other hand, the expression of HLA-G by these cells might be upregulated by the influence of tacrolimus treatment alone, as Levitsky et al. recorded elevated sHLA-G levels in liver graft patients that have been receiving tacrolimus in the induction protocol (Levitsky et al., 2009). Another group indicated the upregulation of HLA-G in response to hydrocortisone derivatives (Akhter et al., 2012). The main point of using immunosuppressive drugs is to induce depletion or inhibition of T cells to allow minimal immune response against graft tissue, this could result in the expansion of other sets of lymphocytes including CD4+ Treg cells which could have potent regulatory effects on the remaining cells (Goldrath et al, 2004; Salama et al, 2003). Tacrolimus's mode of action involves the down regulation of IL-2 via inhibiting mRNA gene expression especially in CD4+ T cells, also, the molecule is a

powerful T cell suppressor as it has been reported that CD4+ and CD8+ interaction with APC could be significantly downregulated (Thomson, Bonham, & Zeevi, 1995). Consequently, IL-10 can suppress the synthesis of IL-2 and INF- γ released by T helper 2 lymphocytes (Mosmann & Coffman, 1987) which could preferentially alter the ratio of T helper 1 and 2 lymphocytes creating an immunosuppressive environment (Thomson et al., 1995).

Despite the presence of immunosuppressive drugs in combined culture treatment, the cells often showed a similar pattern of HLA-G expression compared to the combined drugs without CMV particle treatment. This may indicate the potent role of CMV particles to activate the immune cells and induce HLA-G expression as has been shown in the results of this work. This could emphasise the risk of developing CMV episodes following transplantation. However, the effectiveness of these immunosuppressive drugs may vary according to each individual response and the extent of action could rely on individual susceptibility (Lu et al., 2011).

The data presented here have indicated that Mycophenolate treatment has shown no significant upregulation of HLA-G after culture, possibly due to the potent immunosuppressive effects exerted by this medication on T and B cells, as studies have demonstrated that mycophenolate can suppress the proliferation of B cells and immunoglobulin synthesis in addition to inhibiting cytokine production (Jonsson & Carlsten, 2003). Moreover, Mycophenolate has a prominent influence on inhibiting TNF α and upregulating the release of IL-10 (Allison & Eugui, 2005; Schneider-Gold et al, 2006) which in turn may affect the expression mechanisms of HLA-G on PBMCs. In order to validate the data presented here, further studies may be required as the sample size used in these experiments was low and statistical power could be enhanced by involving larger cohort.

9.8.1. Immunosuppressive drug effects on CD103 expression

Regarding CD103 expression on peripheral blood PBMCs in response to immunosuppressive drug treatment, most of the lymphocyte populations have shown a trend of upregulation of the receptor, especially with prograf treatment. However, only in NK cells was shown to be significantly upregulating CD103. This in part could be explained by virtue of CD103 upregulation being dominated by the influence of CMV treatment rather than the effects of tacrolimus or mycophenolate

induction. The previous work on CD103 in the current study revealed upregulation of this protein on PBMCs in response to CMV induction. The mechanism behind it may involve the upregulation and activation of TGF- β via inducing integrin $\alpha\beta 6$ by CMV which could ultimately lead to the upregulation of CD103 expression on PBMC populations (Kossmann et al., 2003; Michelson et al., 1994a; Munger et al., 1999; Tabata et al., 2008). In addition, the ability of CMV to upregulate TGF- β has been reported in the current research through qPCR after inducing B cells isolated from whole PBMC culture with CMV.

Another potential mechanism involves the ability of Tacrolimus to exert immunosuppressive effects via augmenting TGF- β gene expression (Thomson et al., 1995) and in the presence of CMV, this could enhance expression of CD103 on PBMCs in the peripheral circulation. This process may involve the presence of T reg lymphocytes which could express CD103 and facilitate and/or enhance epithelial receptor E-cadherin adhesion (Annacker et al., 2005; Banz et al., 2003; Lehmann et al., 2002). Another group has revealed that allostimulated CD8⁺ cells express the CD103 receptor and might be potent immunosuppressants capable of releasing IL-10 (Uss et al., 2006). The magnitude of CD103 expression on PBMCs could participate in driving CD103⁺ cells to infiltrate graft tissue and ligate with its ligand receptor E-cadherin present on epithelial cells, hence promoting early graft rejection (Uss et al., 2006; Wever et al., 1998). This could also magnify the role played by CMV in the induction of CD103 expression by PBMCs and the consequence of migration of these cells to renal tissue where they can originate early dysfunction through interaction with E-cadherin on renal epithelial cells. On the other hand, Mycophenolate may induce the production of IL-10 (Allison & Eugui, 2005; Schneider-Gold et al., 2006) which could in turn act on eliciting CD103 expression (Sigmundsdóttir et al., 2004; Uss et al., 2006) by peripheral blood mononuclear cells which may allow infiltration of renal tissues and trigger the rejection process.

The HLA-G expression on CD103⁺ cells described in the current study could be a consequence of the influence of CMV particles in the treatment combination described earlier or due to the synergistic effects exerted by immunosuppressive drugs. Since tacrolimus has been shown to promote HLA-G expression (Levitsky et al., 2009), together with CMV stimulation more HLA-G may be induced on CD103⁺ lymphocytes, hence, a potent suppressor CD4⁺ and CD8⁺ cells could be obtained with potential ability to regulate or inhibit other effector cells. However,

more detailed phenotypic and functional analysis of CD103+HLA-G+ cells induced by CMV and immunosuppressive drugs would be needed to test this in future studies.

9.8.2. Immunosuppressive drug effects on BAFF and BAFF-R expression

Expression of the BAFF protein on various PBMCs showed a trend of augmented levels in different immunosuppressive drug treatments, albeit, no significant upregulation was obtained in all treatments. Other studies have elucidated the effect of CMV on upregulating the molecule as Xu et al. recorded dramatic increment in membrane-bound and soluble BAFF in CMV+ renal transplant patients and that increased sBAFF concentration was associated with elevated viral DNA load (Haiyan Xu et al., 2014). It was suggested that CMV can elicit the production of INF- γ which is considered as a potential stimulator for BAFF expression (Ittah et al., 2006). This suggestion is supported in the current research which described that CMV could induce the release of INF- γ in PBMCs as qPCR experiments have described earlier a significant augmentation of INF- γ in B cells isolated from whole PBMCs culture with CMV antigen. Another possible mechanism for the induction of BAFF on PBMCs is that during CMV infection there is an immense propagation of virions which could be identified by Toll Like Receptor (TLR-9) (Kuenzel et al., 2010) which may provoke BAFF expression on B cells that will activate them and enhance antibody secretion (Abu-Rish et al., 2013).

Increased BAFF expression on PBMCs could implicate the possibility of this protein to elicit tissue rejection in kidney allografts. This has been further supported by some groups who demonstrated elevated levels of BAFF protein expression in patients with autoimmune diseases (Groom et al., 2002; Mariette et al., 2003). Another study has revealed that augmented levels of BAFF were positively correlated with kidney transplant rejection (Thaunat et al., 2008). A possible explanation relies on antibody mediated recognition of either self-antigen or alloantigen which could be induced through antibody mediated responses resulting from switching B cells from IgM to IgG antibodies (Litinskiy et al., 2002). Also, T lymphocyte activity and antibody switching were shown to be influenced by the interaction of BAFF with its ligands (Thibault-Espitia et al., 2012).

However, tacrolimus (Prograf) and Mycophenolate have not manifested significant influence on BAFF expression by most of the PBMC subsets and in all drug combinations CMV had more impact rather than the drugs in each individual treatment group. This could indicate the potent effect imposed by CMV which may override the suppressive properties of the medications, as most of the upregulated BAFF protein observed in treated PBMCs in comparison to control and drug treatment without CMV particles could be derived from CMV upregulation as discussed earlier. This proposition is further supported by Chung et al. who reported a dramatic reduction in the count of immature B cells and their cytokines IL-21 and IL-10 following renal transplantation (Chung et al., 2014). Also this could emphasize the role played by BAFF as a costimulator for T cells in parallel to B cells and this signalling may occur through BAFF-R expression by T cells and may lead to improved T lymphocyte endurance and survival (Ng et al., 2004).

Regarding HLA-G co-expression on BAFF+ PBMCs, immunosuppressant treatments were unable to elicit significant changes in HLA-G apart from CD3+CD56+BAFF+ T cells treated with Prograf and CMV particles. The expression of HLA-G on this population may signify the important role played by these cells to generate immune tolerant cells that may exert their influence via cytokines or cell signalling to other effector cells, however, HLA-G upregulation might be due to CMV particles which was reported earlier (Albayati et al., 2017) and such cells may exhibit both tolerogenic behaviour towards CMV with reduced cytotoxic properties and a deleterious impact on the allograft.

As far as BAFF-R is concerned, almost all the PBMCs treated with the immunosuppressant drug combination did not exhibit significant changes in expression with the exception of CD3+CD56+ T cells in Mycophenolate treatment. Reports have indicated that BAFF-R could be expressed on most developed B cells and it plays a key role in survival and maintenance of these cells in circulation (Woo et al., 2011). However, the receptor is not limited to B lymphocytes only, other PBMCs were found to express the protein like T reg and T lymphocytes (Ye et al., 2004). A suggested mechanism would involve enhancing the generation of nuclear factor (NF- κ B) either directly by CMV particles or indirectly by means of cytokine mediated signalling (Caposio et al, 2004; Yurochko et al, 1995), leading to synthesis and expression of BAFF-R on circulating PBMCs (Woo et al., 2011).

The expression of BAFF-R in CMV+ kidney transplant patients was recorded to be significantly higher than in CMV- patients with elevated levels of IgG in the former group (Haiyan Xu et al., 2014). Additionally, overexpression of this receptor in kidney transplant patients has been shown to be associated with higher incidence of graft malfunction and antibody mediated rejection episodes (Thibault-Espitia et al., 2012; Xu et al., 2009). A possible result of overexpression of BAFF-R on B cells could be to increase the efficiency of BAFF binding with its receptor, enhancing proliferation and improving the efficacy of these activated cells to synthesise and release immunoglobulin which may induce antibody mediated tissue rejection, thus modifying their tolerogenic function (Thibault-Espitia et al., 2012).

With regards to the effects of Prograf and Mycophenolate on BAFF-R expression, Mycophenolate treatment was found to significantly augment the receptor on CD3+CD56+ T cells. This is partially because of the potent suppressing properties of tacrolimus on B cells and their cytokines (Chung et al., 2014) as these cells can play a key role in modifying cytokine release from CD4+ lymphocytes (Blair et al., 2010). Unlike Mycophenolate, this treatment was shown to upregulate the expression of BAFF-R on T cells. The mechanism involving the upregulation is not fully clear and may involve CMV particles which could drive the process of expression of this molecule on B and T cell subsets via the same mechanism mentioned above. Another possibility would be that the upregulation of BAFF could increase expression of BAFF-R and up regulates the molecule on T cells via a BAFF-R mediated pathway.

With respect to HLA-G co-expression on BAFF-R+ cells, only prograf treatment demonstrated significant elevation of HLA-G, especially on CD56+ T cells and CD8+ cells. Once again, the CMV may be the leading cause for HLA-G expression on those BAFF-R+ cells due to the ability of CMV to promote tolerogenic molecules like HLA-G to be expressed allowing the virus to be spared from effector immune cells. It might also be as a synergistic influence of prograf and CMV as both have been reported to induce HLA-G expression (Sheshgiri et al., 2009; Yan et al, 2009).

9.9. Summary and future work

In conclusion, the work presented here has clearly shown that CMV is able to increase the proportions of cells expressing HLA-G in a range of different leucocyte

types from healthy subjects, particularly CD56+ T cells, B cells and monocytes. What is unclear is whether cells initially expressing HLA-G were induced to proliferate, or whether HLA-G expression was induced on cells initially negative for this molecule. This could be tested by labelling cells with CFSE, culturing with CMV and measuring HLA-G expression on proliferated cells by flow cytometry. In the majority of the experiments in this thesis, a CMV strain AD169 antigen extract was used but whole AD169 CMV particles were shown to have similar effects. It would be of greater relevance to repeat these experiments using viable clinical CMV isolates having a complete CMV genome.

Increased expression of HLA-G would potentially enable cells to exert inhibitory influences on any cells expressing ligands for HLA-G, either directly by cell-cell contact or indirectly via secretion of sHLA-G. This may be a further immune evasion strategy in addition to those already established for CMV. Future work could investigate whether the lymphocytes induced to express HLA-G have antigen receptors specific for CMV. In the case of T cells, this could be investigated using HLA tetramers and in the case of B cells using labelled CMV antigens.

In kidney transplant patients, proportions of HLA-G+ cells of several subsets were found to increase post transplantation, and the in vitro experiments indicated that this could be at least partly a result of Prograf and Mycophenolate therapy. It would be of great interest to observe changes in HLA-G expression in transplant patients (or other patients) having an episode of CMV viraemia to ascertain whether CMV reactivation would have an additional effect. Unfortunately, none of the patients followed in this study experienced a CMV episode during the period of study.

An unresolved issue is the effect of HLA-G genotype on HLA-G expression in response to CMV and it was not possible to confirm the previously reported genetic associations between SNPs and levels of expression. This could be tested in future work by using healthy donors of defined HLA-G genotype and comparing levels of HLA-G mRNA with and without CMV antigen treatment using quantitative RT-PCR.

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